Activated Neutrophils and Bacterial Infection the Lung Against Acute Injury Mediated by Adenoviral Augmentation of Elafin Protects

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Adenoviral Augmentation of Elafin Protects the Lung Against Acute Injury Mediated by Activated Neutrophils and Bacterial Infection

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During acute pulmonary infection, tissue injury may be secondary to the effects of bacterial products or to the effects of the host inflammatory response. An attractive strategy for tissue protection in this setting would combine antimicrobial activity with inhibition of human neutrophil elastase (HNE), a key effector of neutrophil-mediated tissue injury. We postulated that genetic augmentation of elafin (an endogenous inhibitor of HNE with intrinsic antimicrobial activity) could protect the lung against acute inflammatory injury without detriment to host defense. A replication-deficient adenovirus encoding elafin cDNA significantly protected A549 cells against the injurious effects of both HNE and whole activated human neutrophils in vitro. Intratracheal replication-deficient adenovirus encoding elafin cDNA significantly protected murine lungs against injury mediated by *Pseudomonas aeruginosa* in vivo. Genetic augmentation of elafin therefore has the capacity to protect the lung against the injurious effects of both bacterial pathogens resistant to conventional antibiotics and activated neutrophils. The Journal of Immunology, 2001, 167: 1778–1786.

Toxic products released by neutrophils, especially human neutrophil elastase (HNE), have been implicated in the pathogenesis of a variety of inflammatory disorders characterized by pulmonary neutrophilia, including cystic fibrosis (CF), non-CF bronchiectasis, emphysema, and bacterial pneumonia (1–4). The therapeutic rationale for depleting circulating neutrophils in such conditions is negated by the propensity for overwhelming sepsis in neutropenic patients and by the observation that mice deficient in neutrophil elastase are predisposed to Gram-negative sepsis (5). This argument applies especially in the context of bacterial pneumonia. In such situations, inhibition of extracellular HNE by agents also possessing antimicrobial properties would be an attractive strategy in attempting to protect the lung from inflammatory injury.

Inhibitors of HNE are thought to comprise part of the human innate immune system. Three distinct antielastases have been described in the human lung: α1-protease inhibitor, secretory leukocyte protease inhibitor (SLPI), and elafin (elastase-specific inhibitor) (6). Elafin (7, 8) is a potent inhibitor of HNE and proteinase 3 produced in the skin (9–11), and in the airways (12), which is up-regulated in response to early inflammatory cytokines such as TNF and IL-1 (13). Elafin, along with SLPI, also shares characteristics with antimicrobial defensin-like molecules in being a low m.w. cationic peptide with the ability to eliminate pulmonary pathogens (14–16).

We therefore hypothesized that local augmentation of elafin by constitutive lung cells would confer protection against inflammatory injury, especially when the lung was challenged by bacterial pathogens. In these studies, we used an adenoaviral gene transfer approach for a number of reasons, including the natural tropism of adenovirus for the respiratory epithelium, the potential to regulate transgene expression using carefully selected promoters (17, 18), and the well-described ability to express antielastases in the lung using adenoviral gene therapy (17, 19). Against this background, we demonstrate for the first time that A549 cells transfected with adenovirus encoding human elafin in vitro protects not only against HNE-induced damage but also against injury caused by primed and activated human neutrophils. We also show for the first time that intrachacheal (i.t.) transfer of adenovirus encoding a human gene with dual antielastase and antimicrobial properties protects murine lungs against acute inflammatory injury caused by *Pseudomonas aeruginosa*, a bacterial pathogen commonly resistant to conventional antibiotics (20, 21). Our findings extend the observed potential for gene therapy strategies in the management of pulmonary injury and infection (22–26) by demonstrating that the protection conferred in vivo was achieved using doses of adenovirus which were not themselves associated with significant vector-induced airway inflammation.

Materials and Methods

**Adenoviral constructs**

The adenoviral constructs used have been described in detail elsewhere (17, 18). In brief, human elafin cDNA (encoding full length elafin) was cloned into pBKH downstream of a 1.4-kb fragment of the murine CMV promoter (27). PDK6 and pBHG10 were used to cotransfect 293 cells.
(which generate the product of the adenoviral E1 region in trans). Homol- 
genous recombination resulted in the generation of E1-; partially E3-deleted 
adeno virus encoding human elastin cDNA (Ad-elafin). The second virus 
used was an Ad-3-deleted adeno virus encoding lacZ (Ad-
lacZ) was constructed in the same manner with the exception that lacZ 
cDNA (under the control of the same murine CMV promoter fragment) 
was cloned in place of elastin cDNA (17, 18).

Preparation of human neutrophils
Fresh citrated whole blood was obtained from healthy volunteers. Neutro-
phils were prepared by dextran sedimentation and Percoll gradient extrac-
tion using a standard technique described elsewhere (28). BSA (Sigma, 
Poole, U.K.) at a final concentration of 1% was added to cell supernatants 
before addition of neutrophils. Neutrophils were activated by the sequential 
addition to cell supernatants of platelet-activating factor (PAF) (Calbio-
chem, Nottingham, U.K.) at a final concentration of 10−9 M, and FMLP (Calbio-
chem), final concentration 10−7 M.

In vitro transfection experiments
A549 cells were used in in vitro transfection experiments. A549 cells are 
derived from bronchioalveolar cell carcinoma (29) and share several phe-
notypic characteristics with type II alveolar epithelial cells (29, 30). Indeed, 
several features of A549 cells have been reproduced in primary cell lines; 
in particular elastin is expressed by pulmonary epithelial cell lines and 
upregulated in response to IL-1 and TNF, features characteristic of A549 cells 
(13, 30).

A549 cells were incubated at 37°C and (5% CO2) in DMEM (Sigma) 
containing 10% FCS (Sigma), penicillin G (final concentration, 100 U/ml; 
Life Technologies, Paisley, U.K.), streptomycin sulfate (final concentra-
tion, 100 μg/ml; Life Technologies), and 1% L-glutamine (final concen-
tration, 2 mM; Life Technologies) and grown to confluence in 24-well 
plates (Corning Costar, High Wycombe, U.K.). Cells were washed with 
PBS (Sigma) and then incubated for 30 min at 37°C with one of three 
treatments; Ad-elafin in DMEM containing 5% FCS; Ad-lacZ in DMEM 
containing 5% FCS; or DMEM containing 5% FCS with no virus added.

Adenovirus was applied at a multiplicity of infection (moi) of 50 PFU 
(except in dose-response experiments where adenovirus was applied over 
the dose range 0–50 PFU). Cells were washed with PBS, and the medium 
was changed to DMEM containing 10% FCS, before incubation overnight 
at 37°C. Cells were washed extensively using PBS and incubated at 37°C 
in serum-free DMEM before the addition of either purified HNE at a final 
concentration of 4 μg/ml (Elastin Products, Owensville, MO) or 2 × 106 
human neutrophils (activated by PAF and FMLP) for 20 min. After 16 h, 
the damage to the monolayer was assessed morphologically by light micros-
copy or by counting the number of A549 cells liberated into the 
supernatant.

In a variation of these experiments, A549 cells were radiolabeled with 
31In, using a variation on methods described previously (31). 31In (Du-
Pont, NEN Life Sciences, Brussels, Belgium) was incubated with 4 × 10−3 
M tropolone (Sigma) for 1 min. 111In (3 μCi) was added to A549 cells 
at the end of the overnight incubation in DMEM containing 10% FCS (final 
concentration of tropolone, 4 × 10−3 M). Damage to the monolayer after 
addition of human neutrophils was assessed 16 h later by measuring ra-
dioactivity in the supernatant using a scintillation analyzer (model 1900TR, 
Canberra Packard, Pangbourne, U.K.). It is well established that neutro-
phils from different healthy individuals may respond very differently to the 
effect of priming agents. In preliminary experiments, 2 × 106 neutrophils, 
activated with 10−7 M PAF and 10−8 M FMLP, caused 30–70% damage to 
untransfected A549 cells at 16 h in the significant majority of individu-
als. A protocol was therefore devised whereby neutrophils causing <30% 
or >70% damage to untransfected monolayers were excluded from analysis. 
In subsequent experiments, 10 healthy volunteers donated neutrophils.
In eight of these the protocol was satisfied (in one of the remaining cases, 
100% of untransfected A549 cells were damaged at 16 h, and in the other 
no untransfected A549 cells were morphologically damaged at 16 h).

To correct for the theoretical risk of nonspecific leakage of 111In from 
A549 cells, radioactivity in supernatants from cells that did not have neu-
rophils added was subtracted from the count in neutrophil-treated cells 
for all conditions studied.

Preparation of P. aeruginosa PAO1
P. aeruginosa PAO1, a well-characterized type strain used in many genetic 
(21) and animal studies, and the first strain of P. aeruginosa to be fully sequen-
ced (22), was inoculated into nutrient broth (Oxoid, Basingstoke, 
U.K.) containing 0.5% yeast extract (Difco, Detroit, MI) and incubated 
overnight at 37°C in an orbital incubator at 200 rpm (3). The culture was 
centrifuged at 4500 rpm at room temperature for 15 min, and the bacteria 
were washed in 0.01 M phosphate buffer, pH 7.0. The bacterial suspension 
used in in vivo experiments was prepared in the same buffer to provide a 
population density of ~2.2 × 107 CFU/ml when cultured on Pseudomo-
nas Isolation Agar (Difco).

In vivo experiments
Female C57BL/6 mice between 6 and 8 wk old were from Harlan Olac 
(Bicester, U.K.). Mice were anesthetized using i.p. avertin (~10 μg 
body weight; avertin comprised 1.25% 2,2,2-tribromoethanol (Aldrich, Gilling-
ham, U.K.) and 2.5% 2-methyl-2-butanol (Sigma)). In all experiments in-
volved i.t. administration, the vocal cords were viewed directly, and a 
blunted catheter was passed beyond them according to methods previously 
described by our group (33). Treatments of known volume were instilled 
directly into the body fluids of the experimental animal. Preliminary 
experiments in which trypsin blue was administered i.t. consistently 
revealed a similar distribution of dye to all lobes of the lung macroscop-
ically (data not shown). All mice were euthanized under anesthesia (using 
avertin) by transection of the abdominal aorta.

In one set of preliminary experiments, four mice that had not received 
any form of i.t. treatment were anesthetized as above and euthanized. 
The lungs and trachea were removed en bloc and bronchoalveolar lavage fluid 
(BALF) was obtained by instillation of two separate aliquots of 250 μl 
sterile PBS. BALF was serially diluted, inoculated onto Pseudomonas 
Isolation Agar, and incubated at 37°C overnight. The remaining BALF was 
centrifuged at 2000 rpm for 10 min at 4°C. The pellet was resuspended in 
PBS; the total cell count was established and cytospins were prepared to 
determine differential cell count. The supernatant was stored at −40°C be-
fore further use.

In a further set of preliminary experiments mice were anesthetized as 
above and received an i.t. instillation of either Ad-elafin (3 × 107 PFU) 
suspended in PBS, Ad-lacZ (3 × 107 PFU) suspended in PBS, or PBS 
alone (n = 4 in each group). Five days later, mice were anesthetized in the 
same way and given i.t. PBS. After 24 h, mice were euthanized as above. 
BALF was prepared to establish P. aeruginosa PAO1 colony counts and 
differential cell counts as described above (with the exception that BALF 
was prepared using aliquots of 250 μl and then 2 μl).5 In the ensuing 
experiments, Ad-elafin (3 × 107 PFU) suspended in PBS, 
Ad-lacZ (3 × 107 PFU) suspended in PBS, or PBS alone were instilled by 
i.t. injection in a final volume of 40 μl (PBS group n = 18, Ad-lacZ group 
n = 20, Ad-elafin group n = 16). Five days later, mice were anesthetized 
in the same way, and a direct i.t. instillation of P. aeruginosa PAO1 was 
administered (~2.2 × 1011 CFU/ml suspended in 0.01 M phosphate buffer; 
final volume, 34 μl). In preliminary experiments, we found that P. aerugi-
 nosa PAO1 at this predetermined dose consistently produced a sublethal 
pneumonia. Twenty-four hours after administration of P. aeruginosa 
PAO1, and the lungs were removed en bloc before fixing 
and storing in 0.01 M phosphate buffer, pH 7.0. The spleen was inoculated onto 
Pseudomonas Isolation Agar and incubated at 37°C over-
night. The spleen was stored in 0.01 M phosphate buffer, pH 7.0. The bacterial suspension 
was washed in 0.01 M phosphate buffer, pH 7.0. The bacterial suspension 
used in in vivo experiments was prepared in the same buffer to provide a 
population density of ~2.2 × 107 CFU/ml when cultured on Pseudomo-

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Levels of human elastin Ag were measured using a sandwich ELISA avail-
able in-house, performed on 96-well plates (Linbro; Flow Laboratories,

4 Other data relating to these control mice have been submitted elsewhere, but the data 
presented in this article do not appear elsewhere.
McLean, VA). In brief, the primary Ab was polyclonal rabbit anti-human elafin Ig, gelatin (BDH, Poole, U.K.) was used as a blocking agent, the sample (or purified recombinant elafin as standard) was applied, and the secondary Ab was biotinylated polyclonal rabbit anti-human elafin Ig (biotin was from Pierce, Rockford, IL). Streptavidin–HPO complex (Sigma) and then chromogenic substrate (2,2’-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); Sigma) were added sequentially and the OD_{550} of the product was quantified using a Dynatech M5000 Plate Reader (Dynex, Bellingham, U.K.).

Elastase activity was measured by applying the elastase-specific chromogenic substrate N-methoxy succinyl- Ala-Ala-Pro-Val-p-nitroanilide (Sigma), and measuring change of OD_{550}. Elastase-inhibitory activity (EIA) was measured by adding sample (serially diluted in buffer (50 mM Tris, 0.1% Triton, 0.5 M sodium chloride, pH 8); final volume, 10 μl) or buffer alone to a known quantity of purified HNE (50 ng in 10 μl) and incubating for 30 min at 37°C before adding 50 μl N-methoxy succinyl-Ala-Ala-Pro-Val-p-nitroanilide and measuring change of OD_{550}. Plots of OD (reflecting HNE activity) against concentration of sample were constructed. As described elsewhere (35), EIA was derived from extrapolation of the curve to the abscissa.

MPO activity was calculated by addition of chromogenic substrate (0.1 mg/ml tetramethylbenzidine (DAKO, Denmark), 0.03% hydrogen peroxide (Sigma) in 0.1 M sodium acetate, pH 4.9) and measurement of the change of OD_{630}. To measure MPO activity in homogenized lung supernatants, samples were diluted in homogenization buffer, and 100-μl aliquots (in triplicate) added to 100 μl chromogenic substrate. To measure MPO activity in BALF, 50-μl aliquots of BALF were treated with 10 μl sodium acetate buffer, pH 4.2, before addition of 100 μl chromogenic substrate.

Protein was measured using the bicinchoninic acid method (Pierce) using purified albumin (Pierce) as standard.

Commercial ELISA kits were used to measure concentrations of murine albumin (Bethyl Laboratories, Montgomery, TX) and human IL-8 (R&D Systems, Abingdon, U.K.).

Measurements of murine keratinocyte-derived chemokine (mKC) concentration in BALF, using an ELISA, were kindly performed by Professor T. Standiford (University of Michigan, Ann Arbor, MI).

Statistics

The parameters studied were not normally distributed, and nonparametric tests were applied. For comparisons involving three groups, the Kruskal-Wallis test was used, and comparisons between pairs of groups were performed using the Mann-Whitney U test. Paired data were studied using Wilcoxon’s rank sum test. Nominal data were compared using the χ² test. Correlations were studied using Spearman’s rank correlation test.

Results

Effects of adenoviral augmentation of elafin on damage mediated by HNE and activated human neutrophils in vitro

Transfection of A549 cells with Ad-elafin resulted in a dose-dependent increase in both secretion of elastin Ag and antielastase activity, indicating that elafin production was efficient and that the elafin produced was functionally active (Fig. 1). Adenoviral transfection did not increase secretion of IL-8 above that from untransfected cells. Indeed Ad-lacZ and Ad-elafin transfection (each at an moi of 50 PFU) resulted in IL-8 levels that were, respectively, 54 and 63% of those from untransfected cells (n = 3; p = 0.05 comparing the three groups).

Ad-elafin significantly protected A549 cells against injury induced by HNE as assessed morphologically (Fig. 2, a–c). This observation was supported by a significant reduction in the number of cells released from pulmonary epithelial monolayers after treatment with HNE (Fig. 2d). The protection conferred was associated with significant and almost complete inhibition of HNE (Fig. 2e).

When human neutrophils, activated with a combination of priming agents (PAF) and secretagogues (IML), were used in place of purified HNE, morphological evidence of protection was again conferred by Ad-elafin (Fig. 3, a and b). This was associated with a reduction in release of 111In from radiolabeled epithelial cells (Fig. 3c). The protection conferred by Ad-elafin was associated with a significant reduction in measured HNE activity (Fig. 3d).

Preliminary in vivo experiments

In mice receiving no i.t. treatments (i.e., normal mice), the median total cell count in BALF was 1.84 × 10⁵, with macrophages comprising >95% of the count in all animals. The remaining cells in BALF were almost exclusively neutrophils, as is characteristic of BALF from normal mice. Median protein concentration in BALF was 0.45 g/L (range, 0.43–0.53 g/L). In mice receiving i.t. vector and/or PBS, very similar trends were observed (Table I). Total cell counts were similar, and the alveolar macrophage was the predominant cell type, with the neutrophil comprising a median of 0.7% of cells in BALF among PBS/PBS mice, 3.4% in Ad-lacZ/PBS mice, and 0.6% of Ad-elafin/PBS mice (no significant difference). Similarly, adenovirus administration itself did not significantly influence the concentration of protein in BALF characteristic of untreated animals or those treated sequentially with vehicle alone (Table I). Therefore, adenovirus administration per se was not associated with significant airway inflammation as assessed by cellular content and protein concentration of BALF. No bacterial growth was obtained on Pseudomonas Isolation Agar plates inoculated with BALF (Table I), blood, homogenized spleen, or homogenized lung from untreated, vehicle-treated, or virus-treated mice, reflecting the absence of either commensal or contaminating P. aeruginosa in our laboratory.

Effects of Ad-elafin transfection on lung injury mediated by P. aeruginosa in vivo

Delivery of Ad-elafin i.t. followed by P. aeruginosa PA01 resulted in a median human elafin concentration in BALF of 8.5 ng/ml (interquartile range, 5.9–10.4 ng/ml). No human elafin was detected in any of the mice given Ad-lacZ (as viral control) or PBS.
A chromogenic substrate assay specific for HNE.

were taken (using a blue filter to optimize contrast), cells in the supernatant were counted, and the residual HNE activity of supernatants was measured using

P. aeruginosa

(as nonviral control) and then P. aeruginosa PAO1, confirming that antielafin IgG does not cross-react with murine or bacterial proteins. Histologically, the administration of P. aeruginosa PAO1 in control mice was associated with a patchy, multilobar pneumonia, associated with extravasation of neutrophils into alveolar airspaces (Fig. 4, a and c) with associated protein leak. These effects were much less pronounced in mice receiving Ad-elafin (Fig. 4b) in which there was a significant reduction in the concentrations of protein and albumin in BALF (Fig. 4d).

The protection conferred by Ad-elafin was associated with a significant reduction in the quantity of bacteria retrieved from BALF (Fig. 4d). Indeed, among the mice receiving Ad-elafin, the concentration of elafin in BALF significantly and inversely correlated with the number of P. aeruginosa PAO1 colonies (log transformed) in BALF ($r = -0.75$; $p = 0.02$). Administration of Ad-elafin was also associated with a significantly lower incidence of bacterial growth in blood (colonies detected in 31% of Ad-elafin mice, 44% of Ad-lacZ mice, and 61% of PBS mice, $p < 0.05$) and spleen (colonies detected in 19% of Ad-elafin mice, 40% of Ad-lacZ mice, and 44% of PBS mice, $p < 0.05$).

In none of the mice studied was elastase activity detected in BALF, reflecting a relative excess of elastase inhibitors in BALF from all animals. However, the total EIA in BALF was lower in the Ad-elafin group than in either of the control groups (Fig. 5).

In addition, BALF from mice receiving Ad-elafin contained significantly lower concentrations of the murine neutrophil chemo-

trophil degranulation products) was also found in mice receiving Ad-elafin (Table II). In keeping with this finding, a trend was observed toward lower concentrations of MPO in homogenized lung (reflecting lung neutrophil count) in mice receiving Ad-elafin (Table II). Indeed, considering all mice together, there was a significant correlation between the concentration of mKC in BALF and the concentration of MPO in homogenized lung ($r = 0.43$, $p < 0.005$). A trend toward lower levels of free MPO in BALF (reflecting neutrophil degranulation products) was also found in mice receiving Ad-elafin (Table II).

Discussion

We believe these data represent the first description of protection against acute lung injury conferred by genetic augmentation of a human pulmonary antielastase/antimicrobial peptide. The findings support and advance the concept that gene therapy may be used to obviate acute lung injury generally and that caused by bacterial infection specifically by manipulation of host defense molecules (22–26).

Our data indicate that Ad-elafin is capable of protecting A549 cells against acute injury mediated by HNE (Fig. 2). Untransfected A549 cells constitutively produce small quantities of antielastases, including elafin (36), but these were overwhelmed by the concentrations of HNE applied here (Fig. 2).

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Our data indicate that Ad-elafin is capable of protecting A549 cells against acute injury mediated by HNE (Fig. 2). Untransfected A549 cells constitutively produce small quantities of antielastases, including elafin (36), but these were overwhelmed by the concentrations of HNE applied here (Fig. 2), resulting in cell damage. In this context, it is interesting both that a similar concentration of HNE may be found in sputum from patients with CF (3) and that

![FIGURE 2. Ad-elafin transfection protects pulmonary epithelial cells against HNE in vitro. A549 cells were treated with Ad-elafin (m0 50 PFU), Ad-lacZ (moi 50 PFU), or medium alone (untransfected cells) for 30 min at 37°C. Cells were washed with PBS to remove residual virus and then incubated overnight at 37°C in medium containing serum. Cells were extensively washed with PBS to remove serum and incubated at 37°C for 48 h in serum-free medium. HNE was then added directly to the supernatant (final concentration, 4 μg/ml) and incubated at 37°C for 16 h. Photomicrographs of cell layers were taken (using a blue filter to optimize contrast), cells in the supernatant were counted, and the residual HNE activity of supernatants was measured using a chromogenic substrate assay specific for HNE. a, Untransfected cells, with no HNE added; b, Ad-lacZ-treated cells, after addition of HNE (appearances of untransfected cells treated with HNE were almost identical); c, Ad-elafin-treated cells, after addition of HNE; d, cell counts in supernatants retrieved after addition of HNE (n = 6). Results were expressed relative to the count in supernatants from untransfected cells, which was regarded as 100% in each experiment. Values represent medians (and interquartile ranges) from the six experiments performed. **, Significantly lower compared with either of the other treatments, $p < 0.01$. e, Residual HNE activity in supernatants (n = 6). Results were expressed relative to the HNE activity in supernatants from untransfected cells, which was regarded as 100% in each experiment. The values shown represent medians (and interquartile ranges) from the six experiments performed. ***, Significantly lower compared with each of the other treatments, $p < 0.005$.](http://www.jimmunol.org/content/1781/1/404.full)
high concentrations of HNE down-regulate constitutive expression of elafin (37). In contrast Ad-elafin transfection augmented elafin secretion sufficiently to protect A549 cells significantly (Fig. 2c).

Extending these findings, we found that Ad-elafin protected A549 cells against human neutrophils maximally activated by a combination of a priming agent and a secretagogue (Fig. 3). Despite the capability of neutrophils to liberate a plethora of potentially cytotoxic agents during activation, the selective inhibition of HNE (and proteinase 3) by elafin resulted in a marked reduction in epithelial injury. This extends our previous observation that HNE is centrally important in neutrophil-mediated tissue injury. Furthermore, the finding that the protection conferred by elafin was associated with functional antielastase activity suggests that, under the experimental conditions described, the methionine in the antielastase site of elafin (38, 39) is resistant to the effects of oxidants associated with functional antielastase activity suggests that, under the experimental conditions described, the methionine in the antielastase site of elafin (38, 39) is resistant to the effects of oxidants.

The epithelial protection associated with Ad-elafin treatment in vitro provided the necessary proof of principle to proceed with gene therapy studies using Ad-elafin in vivo. P. aeruginosa PAO1 was used in these experiments because it is arguably the most studied strain of P. aeruginosa with respect to genetic background (32), virulence, and animal models. Furthermore we have shown that elafin has antimicrobial activity against P. aeruginosa PAO1 in vitro (16). The experimental approach used in this study is worthy of discussion. Firstly the i.t. route used was simple, effective, and minimally invasive (33). This approach efficiently generated human elafin in murine airways; previous immunohistochemical studies have demonstrated human elafin expression in surface epithelia from conducting airways and in alveolar epithelium (data not shown).

Ad-elafin transfection protects pulmonary epithelial cells against activated human neutrophils in vitro. A549 cells were treated as described in Fig. 2 with the exception that incubation in serum-free medium was for 24 h, at which point 1% BSA (final concentration) was added. Freshly prepared human neutrophils (2 × 10⁶) were added directly to the supernatant along with 10⁻⁷ M PAF (final concentration). At 1 h, 10⁻⁷ M fMLP was added (final concentration). Cells were incubated at 37°C for 16 h; photomicrographs were taken (with a blue filter to optimize contrast), and the residual HNE activity of supernatants was measured using a chromogenic substrate assay specific for HNE. In a variation of this experiment, before being washed to remove serum, cells were incubated with 3 × 10⁻³ mCi ¹¹¹In in 4 × 10⁻⁴ M tropolone (final concentration). Cells were extensively washed to remove unincorporated radiolabel and serum and then incubated in serum-free medium for 24 h at 37°C. Neutrophils were added as above, and radiolabel was counted in supernatants at 16 h. a, Ad-lacZ-treated cells, after addition of activated human neutrophils (arrows). Discontinuation of epithelium is seen adjacent to clusters of neutrophils, indicating epithelial injury (appearances of untransfected cells after addition of neutrophils were almost identical). b, Ad-elafin-treated cells, after addition of activated human neutrophils (arrows). c, Radiolabel in supernatants after addition of activated human neutrophils (n = 8). Results were expressed relative to the radiolabel retrieved in supernatants from untransfected cells, which was regarded as 100% in each experiment. Values represent medians (and interquartile ranges) from the four experiments performed. §, Significant difference comparing Ad-elafin with Ad-lacZ, p < 0.05. d, Residual HNE activity in supernatants (n = 4). Results were expressed relative to the HNE activity in supernatants from untransfected cells, which was regarded as 100% in each experiment. Values represent medians (and interquartile ranges) from the eight experiments performed. ∗, Significantly lower compared with each of the other treatments, p < 0.05.
vector per se did not significantly influence outcome in mice receiving bacteria, given that PBS-PAO1 mice had a neutrophil count in BALF of $8.2 \times 10^5$ ($\sim 200$ times the number in mice treated with vector then PBS (Table I)) and a protein concentration of $5.7 \text{ g/L}$ ($\sim 15$ times the concentration in mice treated with vector then PBS (Table I and Fig. 4)). We are not aware of studies demonstrating significant protective effects after doses of adenovirus as low as those used here. Therefore, it appears that gene therapy protocols using sufficiently powerful promoters (17, 18) and low concentrations of vector may, at least in part, be able to circumvent important concerns relating to adenovirus-mediated immune responses (40, 41) and inefficiency of transfection of surface epithelium in the lung (42). Recent advances in vectorology are also likely to improve adenoviral gene delivery to the airways (43).

In our model of murine \textit{P. aeruginosa} lung injury, lungs from control mice showed patchy multilobar consolidation, in keeping with histological appearances in human pneumonia associated with this pathogen (Fig. 4). The appearances were associated with a rise in BALF protein, in turn reflecting disruption of the alveolar-capillary membrane (44, 45). The significant protection against lung injury consequent on Ad-elafin transfection, as demonstrated by BALF protein levels, was also associated with significantly enhanced elimination of bacteria from the airways (Fig. 4) and with a significantly lower incidence of hematogenous bacterial dissemination. Elafin therefore appears to be part of a network of endogenous pulmonary antibiotics which includes the defensins (14, 16, 46).

The neutrophil may potentially have advantageous or detrimental effects in pulmonary inflammation (Ref. 47; reviewed in Ref. 48). Excessive activation of neutrophils certainly appears to be associated with the potential for tissue damage (48). In the mice studied here, the presence of MPO in BALF provided evidence of neutrophil degranulation and by implication neutrophil activation (Table II). Elafin augmentation was associated with a modest reduction in BALF MPO, potentially supporting the inhibition of neutrophil-mediated damage seen in vitro (Table II and Fig. 3). Elafin augmentation was also associated with a reduction in the neutrophil chemokine mKC, and with a corresponding reduction in total lung neutrophils (Table II). Importantly, the relative reduction in pulmonary neutrophilia associated with elafin augmentation did not lead to a significant rise in neutrophil count or protein concentration in BALF.

### Table I. Ad-elafin administration is not associated with a significant rise in neutrophil count or protein concentration in BALF

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<th>White Blood Cell Count ($\times 10^3$)</th>
<th>Neutrophil Count ($\times 10^3$)</th>
<th>Protein in BALF (g/L)</th>
<th>PAO1 Colonies</th>
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<tbody>
<tr>
<td>Ad-elafin/PBS</td>
<td>2.28 (1.74–3.60)</td>
<td>0.015 (0.023)</td>
<td>0.29 (0.25–0.33)</td>
<td>0</td>
</tr>
<tr>
<td>Ad-lacZ/PBS</td>
<td>1.30 (1.02–2.34)</td>
<td>0.045 (0.041–0.060)</td>
<td>0.45 (0.33–0.58)</td>
<td>0</td>
</tr>
<tr>
<td>PBS/PBS</td>
<td>1.46 (0.90–2.54)</td>
<td>0.011 (0.003–0.026)</td>
<td>0.38 (0.22–0.46)</td>
<td>0</td>
</tr>
</tbody>
</table>

* C57BL/6 mice received an i.t. dose of one of the following: Ad-elafin ($3 \times 10^7$ PFU); Ad-lacZ ($3 \times 10^7$ PFU); or PBS. Five days later, PBS was given i.t. The following day the mice were sacrificed, and BALF was retrieved. The total number of white blood cells in BALF was determined, and the neutrophil count was derived by differential cell counts in cytospins. One aliquot of BALF was cultured overnight to determine the number of \textit{P. aeruginosa} PAO1 colonies (indicating commensal or contaminating bacteria), and another was centrifuged and used to determine protein concentration. Results represent medians and interquartile ranges.

**FIGURE 4.** Ad-elafin transfection confers protection against acute lung injury induced by \textit{P. aeruginosa} in vivo. C57BL/6 mice were given an i.t. dose of one of the following: Ad-elafin ($3 \times 10^7$ PFU); Ad-lacZ ($3 \times 10^7$ PFU); or PBS. Five days later, \textit{P. aeruginosa} PAO1 was given i.t. (34 μl of a suspension of $\sim 2.2 \times 10^{11}$ CFU/ml). After 24 h, the mice were sacrificed, and BALF was retrieved. One aliquot of BALF was cultured overnight to determine the number of \textit{P. aeruginosa} PAO1 colonies, and another was centrifuged and used to determine protein and albumin concentrations. In a variation of this experiment, lungs were removed en bloc, fixed in formalin, and examined histologically. a, Histological appearance of lung from a representative mouse treated with PBS and then \textit{P. aeruginosa} PAO1; b, histological appearance of lung from a representative mouse treated with Ad-elafin and then \textit{P. aeruginosa} PAO1; c, histological appearance of lung from a representative mouse treated with Ad-lacZ then \textit{P. aeruginosa} PAO1; d, protein concentration in BALF, albumin concentration in BALF, and \textit{P. aeruginosa} PAO1 colonies in BALF (results represent medians and interquartile ranges). *, Significant difference by Kruskal-Wallis test, $p < 0.05$: when comparing groups directly, for each parameter, the difference between Ad-elafin/PAO1 and PBS/PAO1 was significant, $p < 0.05$; Ad-elafin/PAO1 was significantly different from Ad-lacZ/PAO1 with respect to protein and PAO1 colonies ($p < 0.05$) but not albumin concentration; no significant differences were observed comparing Ad-lacZ/PAO1 and PBS/PAO1.
not preclude tissue protection, despite the observation that neutrophilia correlates with reduced mortality in pulmonary infection caused by *Nocardia* (47).

Interestingly, no free murine elastase activity was detected in BALF from any of the mice studied. This can be attributed to the characteristic elevation of EIA consequent on severe lung injury, comprising part of the acute phase response (49). We calculate that human elafin contributed less than 1% of the EIA of BALF in mice receiving Ad-elafin. Taken together, these findings suggest that a direct anti-neutrophil elastase effect does not explain the protective effect of Ad-elafin, in keeping with the growing body of evidence that pulmonary anti-neutrophil elastases harbor several functions (47). Furthermore, inhibition of *Pseudomonas* elastase (a metalloelastase) does not explain the effects of Ad-elafin in this study, because elafin is an inhibitor of serine proteinases and not metalloproteinases. Against this background, the reduction in EIA associated with Ad-elafin administration (Fig. 5) reflects a significant and appropriate down-regulation in the acute phase response as a consequence of tissue protection conferred by Ad-elafin. Thus, the protective anti-inflammatory effect of elafin is associated with antimicrobial activity, with a reduction in neutrophils in lung tissue, and with a reduction in the characteristic host response to tissue injury.

The relative level of protection provided by Ad-elafin in our in vivo studies is worthy of note. Ad-elafin effected a 40% reduction in BALF protein concentrations as compared with control mice (Fig. 4). Given both the severity of injury in the control mice and the remarkable complexity and redundancy in pulmonary anti-inflammatory responses (6), the degree of protection after one administration of a single human gene is encouraging. Interestingly, a nonsignificant trend also emerged potentially suggesting a weak protective effect for adenovirus over vehicle alone (indeed the Ad-lacZ group was included specifically to control for confounding effects of adenovirus). This was manifest as a trend toward lower levels of IL-8 secretion and greater with cytoprotection (Fig. 2d) in vitro and reduction in albumin concentration and EIA in vivo (Figs. 4 and 5). However, the effect seemed most pronounced with regard to bacterial loads, with strikingly (although not statistically significantly) lower levels of bacteria in BALF (Fig. 4) and blood in Ad-lacZ/PAO1 mice as compared with PBS/PAO1 mice. Although it must be emphasized that these trends did not reach statistical significance (whereas differences between Ad-elafin/PAO1 mice and Ad-lacZ/PAO1 mice generally did attain significance), it is tempting to speculate that adenoviral treatment per se may have contributed in some way to bacterial eradication by priming innate immunity. A similar trend has been observed in studies in which rats received sequential i.t. administrations of adenovirus encoding FcγR and *P. aeruginosa* PAO1; delivery of control adenovirus (a null adenovirus, expressing no foreign transgene) resulted in greater clearance of *Pseudomonas* than did PBS, thus further suggesting stimulation of innate immunity by adenovirus per se (54).

Interestingly, stimulation of adaptive immunity by recombinant null adenoviruses has also been recognized (55), and priming of immune mechanisms has also been attributed to Ad-lacZ in gene therapy protocols directed at lung tumors (56).

Overall, our data provide proof of principle for the prevention of inflammatory lung injury using augmentation of elafin, particularly in the context of infection with antibiotic-resistant pathogens. *P. aeruginosa* PAO1 is classically associated with antibiotic resistance, and the mechanisms responsible have been extensively characterized (21, 32). More specifically, the approach we describe may potentially find application in preventing colonization with *P. aeruginosa* in CF and in the setting of patients at risk of pneumonia attributable to *P. aeruginosa* in intensive care units (57). Nosocomial *Pseudomonas* pneumonia is associated with a high mortality despite conventional treatment, due in part to antibiotic resistance (21, 58). Current opinion suggests that endogenous cationic peptide antibiotics, such as elafin, may be less susceptible to resistance than are conventional antibiotics (59).

In summary, genetic augmentation of elafin proved effective at protecting pulmonary epithelium against neutrophil-mediated injury in vitro and against acute injury induced by *P. aeruginosa* in vivo. These data support the concept that endogenous defense molecules can contribute to innate immunity by protecting tissue.

**Table II.** Administration i.t. of Ad-elafin is associated with a trend toward reduced pulmonary neutrophilia

<table>
<thead>
<tr>
<th></th>
<th>PBS/PAO1</th>
<th>Ad-Elafin/PAO1</th>
<th>Ad-lacZ/PAO1</th>
</tr>
</thead>
<tbody>
<tr>
<td>mKC (ng/ml)</td>
<td>0.25 (0.04–0.78)</td>
<td>0.15 (0.02–0.33)*</td>
<td>0.34 (0.27–1.00)</td>
</tr>
<tr>
<td>Lung MPO (U/mg wet weight)</td>
<td>577 (314–744)</td>
<td>309 (104–797)</td>
<td>625 (326–804)</td>
</tr>
<tr>
<td>Free MPO in BALF (U)</td>
<td>1.13 (0.45–2.28)</td>
<td>0.86 (0.35–1.33)</td>
<td>1.09 (0.31–1.93)</td>
</tr>
</tbody>
</table>

*Whole lungs and BALF were obtained from the mice described in Fig. 4. MPO content of BALF was assessed by adding the chromogenic substrate tetramethylbenzidine and measuring the change in OD<sub>450</sub> as a function of time. The left lung of each animal was retrieved, weighed, and snap frozen. On thawing, lungs were immediately homogenized, and MPO content was assessed as described for that in BALF. In addition, mKC levels in BALF were measured by ELISA. Values represent medians, with interquartile ranges.

*Significant difference comparing group treated with Ad-elafin and then *P. aeruginosa* PAO1 and the group treated with Ad-lacZ and then *P. aeruginosa* PAO1; p < 0.05.
against microbial damage in vivo, and in so doing they suggest novel therapeutic strategies.

Acknowledgments

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lisin, an elastase-specific inhibitor in bronchial secre-


