Increased Anionic Peptide Distribution and Intensity during Progression and Resolution of Bacterial Pneumonia

Amanda J. Fales-Williams, Jack M. Gallup, Rafael Ramirez-Romero, Kim A. Brogden and Mark R. Ackermann


Updated information and services can be found at:
http://cvi.asm.org/content/9/1/28

**REFERENCES**

This article cites 21 articles, 13 of which can be accessed free at: http://cvi.asm.org/content/9/1/28#ref-list-1

**CONTENT ALERTS**

Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), more»

Information about commercial reprint orders: http://journals.asm.org/site/misc/reprints.xhtml
To subscribe to another ASM Journal go to: http://journals.asm.org/site/subscriptions/
Increased Anionic Peptide Distribution and Intensity during Progression and Resolution of Bacterial Pneumonia

Amanda J. Fales-Williams, Jack M. Gallup, Rafael Ramírez-Romero, Kim A. Brogden, and Mark R. Ackermann

Department of Veterinary Pathology, College of Veterinary Medicine, Iowa State University, Ames, Iowa 50011-1250, and Respiratory Diseases of Livestock Research Unit, National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa 50010

Received 6 June 2001/Returned for modification 14 August 2001/Accepted 3 October 2001

Anionic peptides (APs) are small anionic antimicrobial peptides composed of 7 aspartic acid residues and are produced in the lungs of humans, sheep, and cattle. Although expression by epithelial cells of some antimicrobial peptides (e.g., β-defensins) of humans and ruminants is increased in response to acute infection, AP expression is not increased during acute infection, which suggests that the expression of the latter peptide is constitutive. In this study, the degree of AP expression during the progression (acute, subacute, and chronic) of bronchopneumonia was determined. Mannheimia (Pasteurella) haemolytica, a known inducer of bovine β-defensins, was inoculated intrabronchially with a fiber-optic bronchoscope in nine 3-month-old sheep, and tissues were collected at 1, 15, and 45 days postinoculation (p.i.); nine control animals received pyrogen-free saline by the same procedure and were killed at the same time points. In the acute group (1 day p.i.), the lungs had lesions typical of bronchopneumonia and the distribution and intensity of AP immunoreactivity (AP-IR) were similar to those of previous studies (minimal intensity and distribution of AP-IR in bronchiolar epithelial cells). In the subacute group (15 days p.i.), there was prominent hyperplasia of bronchiolar and alveolar epithelial cells, and the chronic group (45 days p.i.) had yet more pronounced hyperplasia. In the subacute and chronic groups, the intensity and distribution of AP-IR in the cytoplasm of hyperplastic bronchiolar and type II alveolar cells were significantly increased compared to those of saline-inoculated and contralateral (noninoculated) lung lobes. Although AP expression appears constitutive, the constitutive production of AP is higher in hyperplastic, less differentiated cells than in fully differentiated, mature cells of the respiratory airways. The increased intensity and distribution of AP-IR in immature (hyperplastic) epithelial cells may be a mechanism by which production of a noninducible antimicrobial is increased temporarily during lesion progression and repair. This increased production of AP by hyperplastic cells may protect the lung against further infection until new, fully differentiated epithelial cells are capable of expressing their own inducible array of antimicrobial peptides.

Antimicrobial peptides serve as a part of the innate immune system of respiratory epithelia, potentially reducing bacterial numbers nonspecifically and therefore limiting bacterial colonization (9, 11, 13). Some antimicrobial peptides are upregulated in response to lipopolysaccharide and other mediators of inflammation (9, 10, 14, 18, 19, 21), while others are produced constitutively (9, 13). Anionic antimicrobial peptides (APs) are small homopolymeric peptides of 7 aspartic acid residues that have been detected in the lungs and bronchoalveolar lavage fluid of humans, sheep, and cattle (5–8). An AP has potent antimicrobial activity but is distinct from many antimicrobial peptides for the following reasons: it is smaller in size, it has an anionic charge due to being a sepaerated aspartate, it has a requirement for zinc as a cofactor for bactericidal activity, and it is present in pulmonary surfactants (3, 5, 7). AP may be a product of posttransitional cleavage of a larger peptide, such as a zymogen (7); however, the identity of the mature protein product from which AP is cleaved has not as yet been determined.

Expression of some antimicrobial peptides by respiratory epithelia such as human β-defensin 2 (20) and bovine β-defensins (lingual antimicrobial peptides and tracheal antimicrobial peptides) (10, 18, 19, 21) increases in the presence of pulmonary infection and/or inflammation; however, expression of these peptides in neonates tends to be limited. Although expression of AP occurs in neonates, AP expression is likely constitutive, because AP distribution and intensity are not increased in the lungs of adult humans with pneumonia, children with or without cystic fibrosis, or cattle with acute pneumonia caused by Mannheimia haemolytica (6, 8). It is our hypothesis that AP expression may increase during chronic phases of pneumonia in immature, regenerative epithelial cells during the repair of lung injury. Increased expression of a constitutively expressed antimicrobial peptide such as AP may protect the lung from reinfection during repair and allow time for the cells to mature in order to express inducible forms of antimicrobial peptides such as the β-defensins. In this study, we assessed the distribution of AP expression in acute (day 1), subacute (day 15), and chronic (day 45) lesions of M. haemolytica infection in the lungs of sheep. M. haemolytica is a known...
inducer in respiratory epithelia of bovine β-defensins (lingual antimicrobial peptides and tracheal antimicrobial peptides) (21), whose presence can result in chronic, regenerative lesions in the lung characterized by hyperplasia of alveolar and bronchiolar epithelial cells (1, 12, 15, 17).

MATERIALS AND METHODS

Experimental animals. Eighteen 3-month-old sheep of both sexes and mixed breeds were obtained from Laboratory Animal Resources, Iowa State University. The animals were maintained in isolation rooms in accordance with the procedures approved by the American Association of Laboratory Animal Care. After 24 h, the animals were randomly assigned to two groups; one group was admin-

istered doses of M. haemolytica (strain 82/25; originally recovered from a natural case of pneumonia Pasteurella multocida) grown in tryptophosphate broth to 10 CFU/ml (24 h). An inoculum of either bacterial broth or pyrogen-free saline (5 ml) was deposited into the right cranial lobe bronchus with a fiber-optic bronchoscope as described previously (2, 4, 16, 17).

Collection of lung samples. Animals were euthanized by an intravenous injection of sodium pentobarbital. Lung tissues were collected from the site of inoculum deposition in the right lungs and additionally from the equivalent site in the noninoculated (left) lungs from those animals that received the bacterial inoculum. Two samples of lung per site were collected. Lung tissues were fixed by immersion in 10% neutral buffered formalin (48 h at room temperature).

Immunohistochemistry. Slides were stained for AP-IR by a previously de-
scribed procedure (6, 8). Briefly, slides were deparaffinized and subjected to antigen retrieval (microwaved to boiling, simmered for 5 min at near-boiling

temperature in citrate buffer [pH 6.0], and then cooled in a –20°C freezer for 15 min and returned to room temperature). The slides were washed in TNTB buffer (0.05 M Tris, 0.85% NaCl, 0.05% Tween 20, 1% bovine serum albumin) and then incubated with the mouse anti-AP immunoglobulin G (IgG) 1G9–1C2 at a 1:1,000 dilution overnight in a humidified chamber at 4°C. 1G9–1C2 was pro-
duced in CFI mice by using the synthetic peptide H-DDDDDDD-OH conju-
gated to keyhole limpet hemocyanin (5, 7). The following morning, the slides were warmed to room temperature, rinsed with TNTB buffer, and incubated for 30 min at room temperature with biotinylated goat anti-mouse IgG at a 1:400 dilution (Vector, Burlingame, Calif.). The slides were rinsed in BioGenex wash buffer, and then biotin-sensitive alkaline phosphatase-conjugated streptavidin (BioGenex, San Ramon, Calif.) was applied for 30 min. The slides were rinsed and then exposed to chromogen (Vector Red) for 10 to 18 min, rinsed, coun-
terstained, and dehydrated. Specificities of antibody affinities with tissue sec-
tions were determined by preincubating the primary antibody, 1G9–1C2, with differing concentrations of up to 0.5 mM synthetic AP (H-DDDDDDD-OH). Inhibition of 1G9–1C2 IR was observed after 1G9–1C2 was preincubated for 45 min with 25 μg of the synthetic target peptide/mg. Other included slides that were not incubated with primary antibody or nonspecific mouse IgG served as negative controls. In all control sections, there was no immunoreactivity except in occasional regions of lungs with small foci of necrotic debris.

Scoring of AP immunohistochemistry. The cellular distribution and intensity of AP-IR were assessed and characterized by light microscopy. The distributions of staining of epithelial cells of airways (bronchi and bronchioli) and alveoli were scored on a subjective scale where 0 indicated no AP-IR, 1 indicated 10 to 30% of epithelial cells had AP-IR, 2 indicated that 10 to 30% of epithelial cells had AP-IR, 3 indicated that 30 to 60% of epithelial cells had AP-IR, and 4 indicated that 80 to 100% of epithelial cells had AP-IR. The intensities of AP-IR in epithelial cells were also assessed and characterized by light microscopy. The intensities with tissue sections were scored on an objective scale where 0 indicated no AP-IR, 1 indicated nuclear staining only; 2 indicated multifocal, mild cytoplasmic, and diffuse nu-
clear staining; 3 indicated diffuse, light red cytoplasmic AP-IR, with or without nuclear staining; and 4 indicated diffuse, bright red cytoplasmic AP-IR.

Specificity of AP monoclonal antibody. (i) Assessment of antibody specificity. Antibody specificity was previously assessed by a competitive enzyme-linked immunosorbent assay (5, 7). Briefly, bovine serum albumin–DDDDDDD-OH conjugate (50 μg of conjugate/well) was used as the adsorbed antigen and dif-
fering graded concentrations (1.0 to 0.002 mM) of H-DDDDDDD-OH, H-
GADDDDDD-DH, H-VDDDDDD-DH, H-TQDDGGK-OH, H-GGEEK-OH, and H-SGSXSOS-OH were used as the competitive antigens in the presence of 1G1–1C2. 1G1–1C2 was specific for peptides with C-terminal Asp residues. The antibody recognized H-GADDDDDD-OH nearly as well as H-DDDDDDD-

OH. However, H-VDDDDDK-OH, H-TQDDGGK-OH, H-GGEEK-OH, and H-
SGXSOS-OH were not recognized.

(ii) Identification of antibody epitopes. The epitope binding site of the mono-
clonal (1G9–1C2) antibody was previously determined in great detail (5, 7). Thirty peptides (7 residues each) corresponding to a single residue frameshift of the sequence were synthesized and then screened simultaneously on a derivat-
ized cellulose sheet (SPOTS; Genosys, The Woodlands, Tex.). Antibody 1G9–
1C2 recognized epitopes that contained greater than 2 C-terminal Asp res-
ides.

Statistical analysis. The results of the subjectively scored slide assays were
analyzed by the Kruskal-Wallis test. All calculations were performed with SAS Institute (Cary, N.C.) statistical software. Results were classified as significant when P was <0.05.

RESULTS

Gross lesions. Animals that received the bacterial inoculum had lesions during the acute, subacute, and chronic stages typical of those caused by M. haemolytica pneumonia. Nonin-
oculated (left) lungs and lungs from all lambs inoculated with pyrogen-free saline (control) lacked gross lesions.

Microscopic lesions. The lungs of animals in the acute group (1 day p.i.) that received bacterial broth had extensive areas of edema, hemorrhage, infiltrates of neutrophils, and necrosis typical of M. haemolytica pneumonia (1, 12, 17).

In the subacute group (15 days p.i.), histologic lesions of lung lobes that had received bacterial inoculation were char-
acterized by (i) large foci of caseous necrotic and pyogranulo-
mataous chronically dilated bronchi surrounded by lobular, sup-
purative bronchial cells indicative of bronchopneumonia; (ii) fibrous connective tissue in alveolar septa; (iii) a marked pro-
iferation of large, cuboidal epithelial cells in bronchiocytes; and (iv) alveoli and infiltrates of lymphocytes and plasma cells in large bronchi. There was an underlying pattern of mild inter-
stitial pneumonia and peribronchiolar lymphoid hyperplasia in all sections of lung examined.

The sections of lungs from the chronic (45 days p.i.) group had differing degrees of chronic changes, including alveolar and bronchiolar fibrosis and proliferation of bronchiolar epi-
theila, accompanied by a marked infiltration of lymphocytes and plasma cells. In one animal, small airways were surround-
ed by fibrous connective tissue that sometimes encroached on tortuous bronchioles or even formed organized masses inside lumena (bronchiolitis fibrosa obliterator). The alveolar and bronchiolar lumina contained occasional clusters of neutro-
phils and cell debris.

The lungs of control animals lacked microscopic lesions. However, three animals had mild hyperplasia of peribronchi-
olar lymphoid tissue, with no other significant changes.

AP-IR. AP-IR was mild in bronchioles of the lung lobes contralateral to inoculated lobes and in the bronchial and bronchiolar epithelial cells of lungs inoculated with saline (Fig. 1 and Tables 1 and 2). AP-IR in lungs from the acute group (1 day p.i.) was similar to that described previously for acute pneumonia of cattle and sheep (A. J. Fales-Williams, K. A. Brogden, E. Huffman, J. M. Gallup, and M. R. Ack-

man, unpublished data). Lung sections from the lambs from the subacute group (15 days p.i.) had diffuse AP-IR in hyper-
plastic bronchiolar epithelial cells lining alveoli and bronchi-
ologies in areas of inflammation. AP-IR was also present in indi-
vidual bronchial and bronchiolar epithelial cells adjacent to the foci of peribronchiolar lymphoid hyperplasia. In the remaining,
noninflamed areas of the lungs, AP-IR was similar to that of the control sections (contralateral and saline-inoculated lungs). AP-IR was present in the cuboidal bronchiolar epithelia in regions of bronchioles exhibiting early fibrosis (Fig. 2A), and prominent AP-IR was present in hyperplastic cells of bronchioles and alveoli (Fig. 2B). In the lungs of the lambs in the chronic group (45 days p.i.), AP-IR was intense in the cytoplasm of the bronchiolar epithelial cells in the foci of bronchiolitis fibrosa obliterans, as well as in cuboidal-to-columnar epithelial cells throughout the foci of lobular bronchopneumonia in one lamb with these histologic changes (Fig. 2C). The intensity of AP-IR in hyperplastic bronchiolar epithelial cells was greater in the chronic group (45 days p.i.) than the subacute pneumonia group (15 days p.i.). In sections of the contralateral lung lobes from infected animals, there were no foci of epithelial hyperplasia, other than multifocal epithelial hyperplasia adjacent to the foci of lymphoid hyperplasia. In both the subacute and chronic pneumonia lesions, there was no AP-IR in hyperplastic bronchiolar epithelia when the tissues were incubated with nonspecific mouse IgG. Although there were distinct visual differences in the intensity of AP-IR in the acute group compared to those of the subacute and chronic groups, there were no statistical differences in the subjective scores as determined by the Kruskal-Wallis test; however, the distribution and intensity of AP-IR were significantly increased in areas of epithelial cell hyperplasia compared to those of areas lacking hyperplastic cells (Table 3). In addition, in all locations where lung tissue showed hyperplastic cells in these animals, AP-IR was intense and in all areas lacking hyperplastic cells, AP-IR intensity was minimal and similar to that of controls. Thus, increased AP-IR intensity and distribution occurred only in regions with hyperplastic cells and was consistently present in such cells.

The time points for euthanasia were chosen as representative of the acute, subacute, and chronic stages of the pneumonic lesions (1, 12, 17). In the present study, all of the changes characteristic of natural infection were present in the lungs of animals from all groups. The lesions of mild hyperplasia of peribronchiolar lymphoid tissue present in lambs of the control group were likely secondary to Mycoplasma ovipneumoniae infection (17); however, these changes were minimally present in both the control and bacterium-inoculated lungs and were not associated with AP-IR.

**DISCUSSION**

The mild bronchial and bronchiolar AP-IR in the lungs of the control animals, as well as in contralateral lung sections from noninoculated animals, and the lack of increased AP-IR in the lung tissues from animals with acute pneumonia were similar to the results for humans, sheep, and cattle in previous studies (6, 8).

In contrast, the expression of AP in the lung tissues of lambs with subacute and chronic pneumonia due to infection with *M. haemolytica* was strikingly increased in the lungs of animals with subacute-to-chronic bacterial pneumonia. The increased AP-IR was located in the cytoplasm of hyperplastic bronchiolar epithelial cells and alveolar type II epithelial cells of all areas of the lungs of the animals with these cells. This finding suggests that, while AP expression is not up-regulated in response to acute inflammation, there is a relative increase in AP accumulation within the proliferative (hyperplastic) cells involved in repair. This increase may be due to either increased basal AP expression, increased cytoplasmic storage, or a lack of release or secretion of AP by these cells. An enhanced constitutive production of AP (or its parent zymogen compound) (5, 7) may be an essential part of the biological activity of hyperplastic regenerative epithelial cells during lesion resolution. The mechanism(s) and relevance of the apparent maturity-dependent expression of AP by epithelial cells are unknown, but AP may serve to protect immature lung epithelial cells from microbial infection during repair and allow them time for additional differentiation to the point where they can express the inducible families of antimicrobial peptides.

Although most epithelial cells in normal lungs exhibit AP expression in the cytoplasm and nucleus, the increased AP in hyperplastic cells is due to cytoplasmic rather than nuclear production. AP expression in the cytoplasm is at a location

**TABLE 1. Distribution and intensity of AP-IR in lung lobes**

<table>
<thead>
<tr>
<th>No. of days p.i.</th>
<th>AP-IR distribution score (range)</th>
<th>AP-IR intensity score (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(no. of sheep)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (3)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>15 (3)</td>
<td>1.333 (1–2)</td>
<td>1.333 (1–2)</td>
</tr>
<tr>
<td>45 (3)</td>
<td>1.667 (1–1.5)</td>
<td>1.333 (1–2)</td>
</tr>
</tbody>
</table>

a Distribution and intensity of AP-IR were determined as outlined in Materials and Methods.
closer to the site of initial microbial adherence and colonization. In addition, the cytoplasm also has organelles (e.g., vesicles and vacuoles) for immediate release or secretion that are clearly lacking in the nucleus. Therefore, preferential AP expression in the cytoplasm rather than the nucleus is another feature that differs from AP expression by nonhyperplastic cells and is consistent with AP’s antimicrobial activity. The identification and characterization of the parent zymogen compound are necessary to understand this and other aspects of AP. Unidentified 31- and 64-kDa proteins show positive AP-IR during Western analysis of sheep lung extract.

In summary, this study demonstrates that the distribution of AP is not altered during the progressive stages of pneumonia in most bronchi and bronchioles; however, in bronchioles and alveoli with hyperplastic epithelial cells, there is a marked increase in the cytoplasmic expression of AP. This expression may be a general characteristic of hyperplastic respiratory epithelial cells and may have a role in protection against additional microbial infections during the resolution and repair of lesions resultant of bronchopneumonia.

ACKNOWLEDGMENTS

We thank E. Huffman and K. DeLong for outstanding technical assistance, Jim Fosse of the ISU Biomedical Communications Department for excellent assistance with photo preparation, and Tammy Benson for help in statistical analysis.

This work was supported in part by USDA/NRI project 99–35204-7681. R. Ramirez-Romero received financial support from CONACYT and Universidad Autónoma de Nuevo León, México.

REFERENCES


tides in bronchoalveolar lavage fluid and in respiratory epithelia of patients with and without cystic fibrosis. Infect. Immun. 67:4256–4259.


