The Journal of Laryngology & Otology (2007), ***121***, *973–978.*

doi:10.1017/S0022215107006184
Printed in the United Kingdom
First published online 26 February 2007

Main Article

Expression and immunolocalisation of antimicrobial peptides within human palatine tonsils

S L Ball*†, G P Siou*†, J A Wilson†, A Howard*, B H Hirst*, J Hall*

Abstract

Background: Recurrent acute tonsillitis is one of the most frequent ENT referrals, yet its pathogenesis remains poorly understood, and tonsillectomy still costs the National Health Service more than £60 000 000 annually. Antimicrobial cationic peptides are components of the innate immune system. They are generally small, highly positively charged peptides with broad spectrum antimicrobial activity which function as the body's 'natural antibiotics'. The role of antimicrobial cationic peptides in the susceptibility of patients to recurrent acute tonsillitis is unknown.

Aims: To characterise and compare antimicrobial cationic peptide expression and localisation in human palatine tonsils from control subjects and recurrent acute tonsillitis patients, and to assess the potential role of these peptides in the pathogenesis of tonsillitis.

Methods: Palatine tonsils were harvested with informed consent from 19 recurrent acute tonsillitis patients and from five control subjects undergoing tonsillectomy for sleep disorders. Total ribonucleic acid was isolated and antimicrobial cationic peptide expression was characterised using reverse transcription polymerase chain reaction. Fluorescent immunohistochemical techniques were used to localise antimicrobial cationic peptides within fresh frozen tonsil sections.

Results: Using molecular analyses, the palatine tonsils from control and recurrent acute tonsillitis subjects were confirmed as a site of expression of the antimicrobial cationic peptides human β-defensin 1–3, LL-37 (cathelicidin) and Liver expressed antimicrobial peptide-1 (LEAP-1). We also demonstrated for the first time the expression of Liver expressed antimicrobial peptide-2 (LEAP-2). Our analyses indicated that all six antimicrobial cationic peptides were expressed in all 26 tonsil samples. Immunohistochemical staining indicated that the antimicrobial cationic peptides were localised to the tonsil surface and crypt epithelium. However, the surface epithelium of tonsils from recurrent acute tonsillitis patients showed reduced amounts of antimicrobial peptides human β-defensins 1 and 3, and LL-37, compared with healthy controls.

Conclusion: The tonsil epithelium synthesises an array of antimicrobial cationic peptides which function as host defence. Preliminary immunohistochemical data suggest that the surface epithelium of tonsils from recurrent acute tonsillitis patients contains reduced amounts of such peptides, which may increase these patients' susceptibility to infection.

Key words: Tonsil; Tonsillitis; Antimicrobial Cationic Peptides

Introduction

The palatine tonsils are part of the lympho-epithelial tissue located at the common openings of the gastro-intestinal and respiratory tract.[1] Consequently, they play a key role in initiating innate, cellular and humoral immunity at local and systemic levels.

In humans, each palatine tonsil is composed of lymphoid material covered by a stratified, non-keratinising epithelium. As regards innate host defences, epithelia not only provide a physical barrier to invading micro-organisms, but also act through the synthesis of an array of specific peptides, which function as the body's 'natural antibiotics'.[2] These peptides are collectively termed antimicrobial cationic peptides. In general, antimicrobial cationic peptides are small (1–10 kDa), structurally diverse molecules which display broad spectrum antimicrobial activity against a range of bacteria, fungi and enveloped viruses.[3] Their selective targeting of microbes is attributed to the composition of the host antimicrobial cationic peptide molecules, which tend to be positively charged (i.e. cationic), while microbial membranes carry a negative charge. Once bound to microbes, these predominantly

From the *Epithelial Research Group, Institute for Cell & Molecular Biosciences, and the †School of Surgical & Reproductive Sciences, Faculty of Medical Sciences, Newcastle University, Newcastle upon Tyne, UK.
Accepted for publication: 14 November 2006.

TABLE I

OLIGONUCLEOTIDE PRIMERS USED FOR RT-PCR ANALYSES OF AMPS, AND PREDICTED SIZES OF CDNA PRODUCTS

Gene	Primer sequence		Product size (bp)
	Forward 5′–3′	Reverse 5′–3′	
hβD-1	CCATGAGAACTTCCTACCTTC	GTCACTCCCAGCTCACTTG	221
hβD-2	GTGAAGCTCCCAGCCATCAG	GATTGCGTATCTTTGGACACC	325
hβD-3	GTTCCAGGTCATGGAGGAATC	CAACACTCTCGTCATGTTTCAG	180
LL-37	CATGAAGACCCAAAGGGATG	CACACTAGGACTCTGTCCTG	518
LEAP-1	GTCACCAGTGGCTCTGTTTTC	GTCTTGCAGCACATCCCACAC	191
LEAP-2*	CAAGATGTGGCACCTCAAAC	CAAGATGTGGCACCTCAAAC	284, 482, 802

*LEAP-2 has three potential complementary deoxyribonucleic acid (cDNA) products, dependent upon transcript splicing: 284 bp fully spliced; 482 bp retaining intron 1; and 802 bp retaining introns 1 and 2. RT-PCR = reverse transcription polymerase chain reaction; AMP = antimicrobial peptide; hβD = human β-defensin; C = Cytosine; A = adenosine; T = thymine; G = guanine

hydrophobic peptides integrate into the microbial membranes, causing depolarisation and death.[4] This killing mechanism allows microbes to be destroyed rapidly, in many cases preventing the onset of infection.[5] In addition to their direct antimicrobial function, these effector molecules have recently been proposed to play a role in mediating inflammation through the stimulation of chemotaxis and wound repair.[6]

The microbes most frequently associated with tonsillitis are the group A streptococci. These pathogens can survive naturally in the oropharynx but are prevented from colonising and causing infection by the activities of the normal oropharyngeal flora.[7]

In acute tonsillitis, local innate defences have been shown to change, with reported increases in the antimicrobial proteins, lysozyme and lactoferrin.[8] However, the extent to which other agents of innate immunity contribute, including the antimicrobial cationic peptides, is less well known. The antimicrobial cationic peptide profile of tonsils is not well documented, either in recurrent acute tonsillitis patients or in normal controls.

Thus, in order to investigate the role of antimicrobial cationic peptides in the pathogenesis of tonsillitis, we compared tonsil antimicrobial cationic peptide profiles and expression patterns in both recurrent acute tonsillitis patients and control subjects, using molecular and immunohistochemical techniques.

Materials and methods

Tissue preparation

Ethical approval was granted prospectively for the use of human tonsils removed from patients undergoing routine tonsillectomy. Tonsils were removed from patients undergoing tonsillectomy because of either recurrent tonsillitis or idiopathic, obstructive tonsillar hypertrophy (with no history of recurrent tonsillitis). Tonsils were either kept whole or immediately dissected to separate the surface epithelium from underlying lymphoid tissue. In both cases, tissues were snap frozen in liquid nitrogen within five minutes of removal from the patient.

Reverse transcription polymerase chain reaction

Total and epithelial ribonucleic acid (RNA) was isolated from the tonsils (Promega RNA Isolation System, Promega, Southampton, UK) and subjected to reverse transcription using random hexamer primers. To amplify the antimicrobial cationic peptides, complementary deoxyribonucleic acid (cDNA) primers designed to cross the intron/exon boundaries of the respective gene were used (Table I), and for each RNA sample, a reverse transcription negative control was generated in which reverse transcriptase was omitted. Polymerase chain reaction conditions included 15 minutes at 95°C, 35 cycles of 95°C for 30 seconds, 58°C for 30 seconds (55°C for antimicrobial peptide type LL-37) and 72°C for 1 minute, followed by a final extension step of 10 minutes at 72°C. The cDNA products were separated on 1 per cent agarose ethidium bromide gels and visualised using ultraviolet light. Product optical density was determined on an AlphaImager 1200 gel documentation and analysis system (Flowgen, Nottingham, UK). All cDNA products were verified by sequencing.

Immunofluorescent staining

Seven-micrometre frozen sections of tonsil, mounted on poly-L-lysine coated slides, were generated by the Department of Histopathology, Royal Victoria Infirmary, Newcastle upon Type. Sections were washed in phosphate-buffered saline, fixed in 2 per cent paraformaldehyde, permeabilised using 0.1 per cent Triton X-100 in phosphate-buffered saline, and incubated in 10 per cent serum to block any non-specific protein binding. Sections were incubated overnight at 4°C with 5 μg/ml dilutions (in phosphate-buffered saline) of the respective primary antibody, including either rabbit anti-human β-defensin 1 (Autogen Bioclear, Colne, Wiltshire, UK), goat anti-human β-defensin 2, (Peprotech EC, London, UK), rabbit anti-human β-defensin 3 (Abcam, Cambridge, UK) or rabbit anti-human LL-37 (Phoenix Pharmaceuticals, Karlsruhe, Germany). After overnight incubation, any unbound primary antibody was removed by washing in phosphate-buffered saline. For each staining protocol, negative controls were performed in which the primary antibody was omitted. Secondary antibodies (goat anti-rabbit Tetramethyl Rhodamine Iso-thiocyanate, rabbit anti-goat Tetramethyl Rhodamine Iso-thiocyanate (Sigma Poole, UK)

https://doi.org/10.1017/S0022215107006184 Published online by Cambridge University Press

and goat anti-rabbit Fluorescein Isothiocyanate (Chemicon, Chandlers Ford, Hamps, UK)), diluted to one in 50 in phosphate-buffered saline, were incubated for 1 hour at room temperature and any unbound material was removed by washing. The slides were air-dried for 5 minutes, mounted with Vectashield (Vector Laboratories, Burlingame, California, USA), and imaged on a Leica TCS-NT laser scanning confocal microscope (Leica, Milton Keynes, UK).

Results

Reverse transcription polymerase chain reaction analyses of RNA extracted from whole palatine tonsils (data not shown) and surface epithelium identified cDNAs consistent with the expression of an array of antimicrobial cationic peptides, including the human β-defensin 1–3, LL-37, LEAP-1 and LEAP-2 (Figure 1). In the case of LEAP-2, multiple polymerase chain reaction products were detected, comparable to observations reported from other tissues.[9] The DNA sequence analyses of the encoded cDNA products from control subjects and recurrent acute tonsillitis patients provided identical matches to published sequence data.

To determine the relative expression levels of antimicrobial cationic peptide transcripts, comparing the surface epithelium and the lymphoid interior of paired tonsils, RNA prepared from dissected tonsillar material (from recurrent acute tonsillitis patients and control subjects) was subjected to semi-quantitative reverse transcription polymerase chain reaction analyses. Human β-defensin 1–3, LL-37 and LEAP-1 gene expression were increased four- to 12-fold in the stratified epithelium compared with the lymphoid interior. However, the levels of LEAP-2 expression were comparable in the epithelial and lymphoid tissues (Table II; Figure 2).

TABLE II

RELATIVE EXPRESSION OF AMP TRANSCRIPTS IN TONSIL SURFACE EPITHELIUM VS LYMPHOID INTERIOR

AMP	Relative expression*
hβD-1	× 5.5
hβD-2	× 4
hβD-3	× 9
LL-37	× 6.5
LEAP-1	× 12
LEAP-2	
− 802 bp cDNA product	× 1
− 482 bp cDNA product	× 2
− 284 bp cDNA product	× 1.5

*Comparing tonsil epithelium to lymphoid interior. AMP = antimicrobial peptide; hβD = human β-defensin; cDNA = complementary deoxyribonucleic acid

Immunolocalisation of antimicrobial cationic peptides

To support the molecular data and to provide evidence for antimicrobial cationic peptide synthesis in the tonsil, frozen sections were immunohistochemically

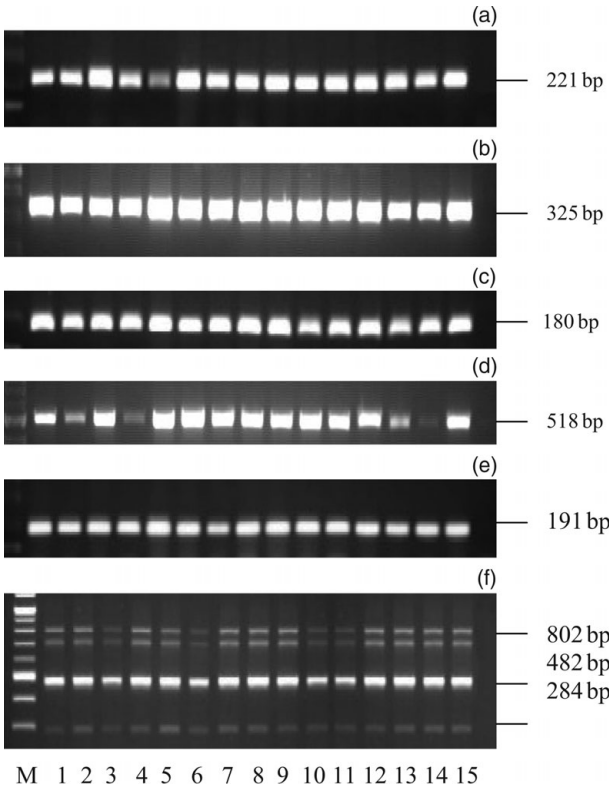

FIG. 1

Reverse transcription polymerase chain reaction of ribonucleic acid isolated from tonsil surface epithelium using specific primers for: (a) human β-defensin (hβD) 1; (b) hβD2; (c) hβD3; (d) LL-37; (e) LEAP-1; and (f) LEAP-2. M = deoxyribonucleic acid marker; lanes 1–13 = tonsillar material from recurrent acute tonsillitis patients; lanes 14–15 = tonsillar material from control subjects

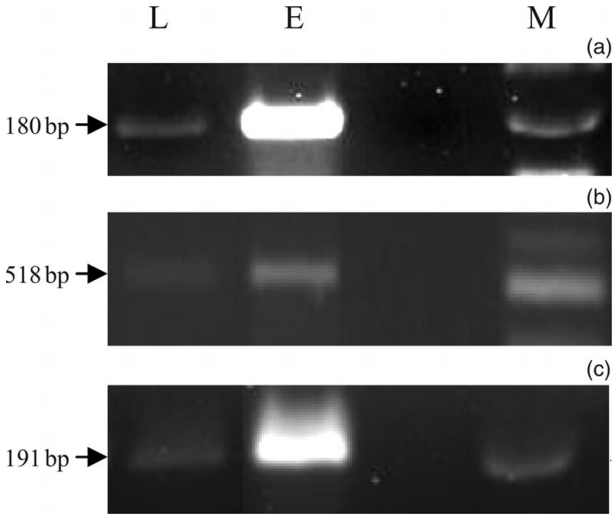

FIG. 2

Reverse transcription polymerase chain reaction of ribonucleic acid (RNA) isolated from lymphoid interior (L) and surface epithelium (E) of the same tonsil using specific primers for: (a) human β-defensin 3; (b) LL-37; and (c) LEAP-1. To enable relative quantification of template and sample RNA concentrations, all reactions were measured in the linear exponential phase, 15 cycles for the 18S ribosomal RNA primer pair and 30 cycles for each antimicrobial primer pair. M = deoxyribonucleic acid marker.

https://doi.org/10.1017/S0022215107006184 Published online by Cambridge University Press

analysed, using antibodies to human β-defensin 1–3 and LL-37.

Immunofluorescent images of human β-defensin 1 stained tonsil tissues taken from a healthy control and from a recurrent acute tonsillitis patient are shown in Figure 3(c) and 3(d). In both cases, the peptide is localised to the surface epithelium. However, the typical staining pattern of tonsils from control patients indicated increased intensity of human β-defensin 1 staining in the outer two or three cell layers of the stratified non-keratinised epithelium, whereas in tonsils from recurrent acute tonsillitis patients the epithelial staining appeared more uniform. Human β-defensin 2 staining (Figure 3e and 3f) also demonstrated the epithelium as the site of maximal expression, with only faint staining in the lymphoid interior. In contrast to human β-defensin 1, however, the human β-defensin 2 staining of the outermost layer of stratified epithelium demonstrated less marked difference between the control and recurrent acute tonsillitis groups. In keeping with the human β-defensin 1 and 2 staining patterns, human β-defensin 3 was localised to the surface epithelial layers, although dense staining in the outer squamous layers of the tonsils from the control subjects was again observed (Figure 3 g and 3 h). Staining with antibody to the cathelicidin LL-37 also localised this peptide to the tonsil epithelium (Figure 3i and 3j) and appeared stronger in the outer few squamous layers of the luminal surface in tissues from control subjects (as was the case with the human β-defensins 1 and 3).

Discussion

In agreement with other authors,[10–12] we found that the tonsil is, as predicted by its location in the mouth, a prominent site of innate defence, with the synthesis of a number of antimicrobial cationic peptides, including the human β-defensin 1–3, the cathelicidin LL-37 and Liver expressed antimicrobial (LEAP) molecules. Moreover, this array of antimicrobial cationic peptides was detected in the tonsillar epithelia of both control subjects and recurrent acute tonsillitis patients, suggesting that these peptides are synthesised in both groups, functioning presumably to limit the adhesion and invasion of pathogenic and commensal bacteria.

The mouth per se appears to be a prominent site of β-defensin expression, with the constitutive expression of human β-defensin 1 reported in gingival tissues[13] and the up-regulation of human β-defensin 2 observed in primary oral cells in response to fungal infection.[14] A previous study reported neither human β-defensin 2 nor human β-defensin 3 expression to be up-regulated in tonsils from patients with recurrent acute tonsillitis.[12] Our molecular analyses support these observations, but our immunohistochemical data are indicative of increased concentrations of human β-defensin 1 and 3 peptides in the outermost layers of the stratified epithelium of control subject tonsils. These data, therefore, suggest that reduced amounts of the host defence peptides are localised to the

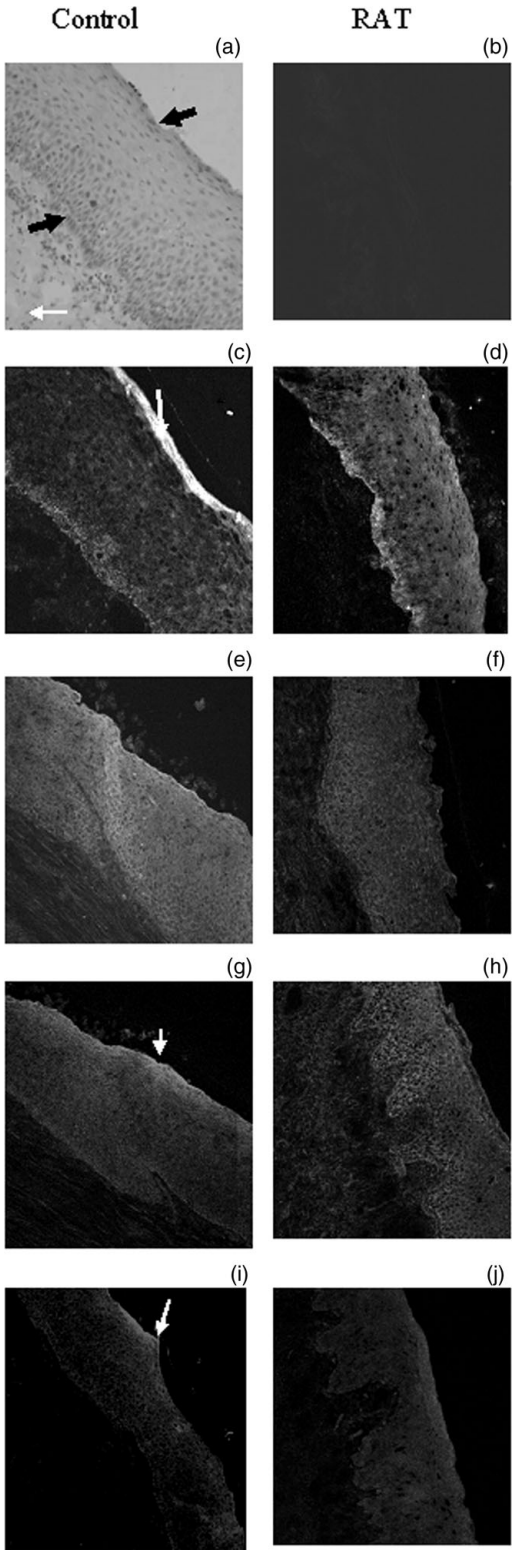

FIG. 3

Immunohistochemical staining of frozen tonsil samples from control subjects (a, c, e, g, i) and recurrent acute tonsillitis patients (b, d, f, h, j). (a) Staining with H&E (black arrows indicate tonsil epithelium, white arrow indicates lymphoid interior). (b) No primary antibody control. (c)–(j) Primary antibody staining of tonsil specific to human β-defensin (hβD) 1 (c, d), hβD2 (e, f), hβD3 (g, h) and LL-37 (i, j) peptides. Increased staining is seen in the outer two or three cell layers of the stratified non-keratinised epithelium (white arrows) in (c), (g) and (i) (×20).

https://doi.org/10.1017/S0022215107006184 Published online by Cambridge University Press

epithelial surface of tonsils excised from recurrent acute tonsillitis patients, thus presumably increasing the susceptibility of the tonsil epithelium to microbial assault.

The cathelicidin host defence peptides are also known to be involved in defence of the oral cavity, with the mouse homologue of LL-37, Cathelicidin related antimicrobial peptide (CRAMP), being expressed in salivary glands and oral mucosa and LL-37 being detected in human saliva.[15] We identified LL-37 transcript expression and peptide synthesis in the tonsillar epithelia of control subjects and recurrent acute tonsillitis patients, with peptide staining patterns again indicative of reduced amounts of the antimicrobial compound in the surface epithelia of tonsils from recurrent acute tonsillitis patients. In contrast to these data, Song *et al.* (2006) reported reduced levels of LL-37 expression in the tonsils of control subjects; in fact, these authors were unable to detect LL-37 transcripts, compared with samples from patients with recurrent throat infections.[16] These contradictory observations are difficult to reconcile. However, they may in part be explained by chronic tonsillar inflammation, associated with the non-specific recurrent throat infections of the patients, causing generalised oropharyngeal up-regulation of mucosal defences and increased LL-37 levels.

The tonsillar expression pattern of the novel LEAP-2 antimicrobial cationic peptide appeared unique, with transcripts divided equally between the tonsil epithelium and lymphoid tissues. This contrasted markedly with the mainly epithelial expression patterns of the human β-defensin 1–3, LL-37 and LEAP-1. Furthermore, LEAP-2 expression was also characterised by multiple transcripts, indicative of the synthesis of an array of peptides of this compound. While the antimicrobial activity of LEAP-2 has been reported against gram-positive microbes,[9] the roles of the additional peptides have yet to be established.

- **Antimicrobial peptides function as the body's 'natural antibiotics'**
- **This study established the antimicrobial peptide profile of tonsils from control subjects and patients suffering recurrent acute tonsillitis**
- **Molecular and immunological studies indicated that the array of antimicrobial peptides expressed and synthesised by tonsils did not differ, comparing control subjects with tonsillitis patients**
- **Results indicated reduced concentrations of antimicrobial peptides in the stratified epithelium of patients with recurrent acute tonsillitis; this may contribute to the increased susceptibility of these patients to infection**

These data provide support for the theory that the tonsillar epithelia of recurrent acute tonsillitis patients synthesise an array of antimicrobial peptides comparable with those of control subjects. Moreover, analyses of the cDNA sequences encoding the antimicrobial cationic peptides from all subjects indicated identical matches to published DNA sequence data. This suggests that patient susceptibility to recurrent acute tonsillitis was probably not a consequence of peptide amino acid mutations resulting in either reduced peptide activity or inactive peptides. However, our immunohistochemical data suggest that the tonsillar surface epithelia of recurrent acute tonsillitis patients contain reduced amounts of human β-defensins 1 and 3 and LL-37 peptides, and it is feasible that this contributes to these patients' increased susceptibility to infection.

It is also possible that the altered antimicrobial cationic peptide concentrations in recurrent acute tonsillitis patients affect their normal oropharyngeal flora, with the altered balance resulting in increased susceptibility of the tonsils to infection. In support of this theory, Crohn's disease, associated with chronic inflammation of the gastrointestinal tract, is thought to be triggered by changes in the balance of intestinal microbiota.[4] Moreover, a group of susceptible Crohn's disease patients carry a mutation in their *NOD2* gene, which encodes an intracellular bacterial pattern-recognition receptor and is associated with reduced levels of defensins. In view of the parallels with recurrent acute tonsillitis, it would be interesting to investigate whether such a similar signalling defect operates in recurrent acute tonsillitis patients. Further studies to quantify tonsillar antimicrobial cationic peptide concentrations will help address such issues.

Acknowledgements

The Journal of Laryngology & Otology provided a bursary for SLB; GPS and AH were supported by the Wellcome Trust.

References

1 Nave H, Gebert A, Pabst R. Morphology and immunology of the human palatine tonsil. *Anat Embryol (Berl)* 2001; **204**:367–73
2 Lehrer RI, Ganz T. Defensins of vertebrate animals. *Curr Opin Immunol* 2002;**14**:96–102
3 Hancock RE, Diamond G. The role of cationic antimicrobial peptides in innate host defences. *Trends Microbiol* 2000;**8**:402–10
4 Wehkamp J, Fellermann K, Herrlinger KR, Bevins CL, Stange EF. Mechanisms of disease: defensins in gastrointestinal diseases. *Nat Clin Pract Gastroenterol Hepatol* 2005;**2**:406–15
5 Brown KL, Hancock RE. Cationic host defense (antimicrobial) peptides. *Curr Opin Immunol* 2006;**18**:24–30
6 Bowdish DM, Davidson DJ, Hancock RE. Immunomodulatory properties of defensins and cathelicidins. *Curr Top Microbiol Immunol* 2006;**306**:27–66
7 Brook I. The role of beta-lactamase producing bacteria and bacterial interference in streptococcal tonsillitis. *Int J Antimicrob Agents* 2001;**17**:439–42
8 Stenfors LE, Bye HM, Raisanen S. Noticeable differences in bacterial defence on tonsillar surfaces between bacteria-induced and virus-induced acute tonsillitis. *Int J Pediatr Otorhinolaryngol* 2003;**67**:1075–82
9 Krause A, Sillard R, Kleemeier B, Kluver E, Maronde E, Conejo-Garcia JR *et al.* Isolation and biochemical characterization of LEAP-2, a novel blood peptide expressed in the liver. *Protein Sci* 2003;**12**:143–52

10 Chae SW, Lee SH, Cho JH, Lee HM, Choi G, Hwang SJ. Expression of human beta-defensin 1 mRNA in human palatine tonsil. *Acta Otolaryngol* 2001;**121**:414–18
11 Harder J, Bartels J, Christophers E, Schroder JM. Isolation and characterization of human beta-defensin-3, a novel human inducible peptide antibiotic. *J Biol Chem* 2001; **276**:5707–13
12 Claeys S, de Belder T, Holtappels G, Gevaert P, Verhasselt B, van Cauwenbergr P *et al*. Human beta-defensins and toll-like receptors in the upper airway. *Allergy* 2003;**58**: 748–53
13 Krisanaprakornkit S, Weinberg A, Perez CN, Dale BA. Expression of the peptide antibiotic human beta-defensin 1 in cultured gingival epithelial cells and gingival tissue. *Infect Immun* 1998;**66**:4222–8
14 Meyer JE, Harder J, Gorogh T, Weise JB, Schubert S, Janssen D *et al*. Human beta-defensin-2 in oral cancer with opportunistic Candida infection. *Anticancer Res* 2004;**24**:1025–30
15 Murakami M, Ohtake T, Dorschner RA, Gallo RL. Cathelicidin antimicrobial peptides are expressed in salivary glands and saliva. *J Dent Res* 2002;**81**:845–50
16 Song JJ, Hwang KS, Woo JS, Chae SW, Cho JG, Kang HE *et al*. Expression of cathelicidin in recurrent throat infection. *Int J Pediatr Otorhinolaryngol* 2006;**70**:487–92

Address for correspondence:
Dr Judith Hall,
Institute for Cell & Molecular Biosciences,
Faculty of Medical Sciences,
Newcastle University,
Newcastle upon Tyne NE2 4HH, UK.

Fax: 44 (0) 191 2227424
E-mail: judith.hall@ncl.ac.uk

Dr J Hall takes responsibility for the integrity of the content of the paper.
Competing interests: None declared