molecular oral microbiology

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Streptococcus pyogenes infection of tonsil explants is associated with a human β-defensin 1 response from control but not recurrent acute tonsillitis patients

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SUMMARY

Host defence peptides (HDP), including the defensins and hCAP-18, function as part of the innate immune defences, protecting the host epithelia from microbial attachment and invasion. Recurrent acute tonsillitis (RAT), in which patients suffer repeated symptomatic tonsil infections, is linked to *Streptococcus pyogenes*, a group A streptococcus, and may reflect the impaired expression of such peptides. To address this, the defensin and hCAP-18 messenger RNA expression profiles of 54 tonsils excised from control and RAT patients undergoing tonsillectomy were quantified and compared. Marked variation in expression was observed between individuals from the two groups, but statistically no significant differences were identified, suggesting that at the time of surgery the tonsil epithelial HDP barrier was not compromised in RAT subjects. Surgical removal of the tonsils occurs in a quiescent phase of disease, and so to assess the effects of an active bacterial infection, HaCaT cells an *in vitro* model of the tonsil epithelium, and explants of patient tonsils maintained *in vitro* were challenged with *S. pyogenes*. The HaCaT data supported the reduced expression of *hCAP-18/LL-37, human β-defensin 1 (HBD1;* $P < 0.01$) and *HBD2* ($P < 0.05$), consistent with decreased protection of the epithelial barrier. The tonsil explant data, although not as definitive, showed similar trends apart from *HBD1* expression, which in the control tonsils but not the RAT patient tonsils was characterized by increased expression ($P < 0.01$). These data suggest that *in vivo* HBD1 may play a critical role in protecting the tonsil epithelia from *S. pyogenes*.

INTRODUCTION

Streptococcus pyogenes, also known as group A streptococcus, is responsible for a series of human diseases ranging from localized infections including tonsillitis and impetigo, to serious life-threatening conditions such as necrotizing fasciitis and rheumatic fever. The global impact of such diseases has been estimated at more than half a million deaths per year, which places this bacterium among the world's major human pathogens (Carapetis *et al.*, 2005). Rarely life-threatening, but among the most frequent of human infectious diseases affecting both adults and children, is tonsillitis. The aetiology of the disease, in which sufferers present with swollen and painful

palatine tonsils, is commonly linked with the presence of *S. pyogenes.*

Streptococcus pyogenes is predominantly an extracellular pathogen and a number of cell surface components have been reported to function in its adherence to human cells (Jenkinson & Lamont, 1997). Certain M protein serotypes, including the M1 strain, are of interest medically because of their association with pharyngitis (Muotiala *et al.*, 1997) and throat infections (Colman *et al.*, 1993), further substantiated by mice studies in which intranasal innoculations with the M1 serotype actually cause throat infections (Lukomski *et al.*, 2000). Recent studies using human explants have shown that pili or fimbriae, which are bacterial cell surface structures of the M1 *S. pyogenes* strain SF370, enhance the adhesion of the serotype to tonsil and presumably play an important role in the initial stages of a tonsil infection (Abbot *et al.*, 2007).

Treatment of a tonsil infection is, in the first instance, with antibiotics prescribed to stop the infection and to reduce swelling. When the infection is severe or recurrent and no longer managed by antibiotic treatment the tonsils are removed. Although tonsillectomy is still the single most common operation in Ear, Nose and Throat units (Little & Williamson, 1996) an increasing number of adults and children are hospitalized annually for throat infections. In 2000–2001, in England, there were 30,942 tonsil-related admissions for medical treatments. By 2008–2009, the figure had risen to 43,641 medical admissions for throat symptoms, an increase of over 41% (12,700 admissions) in 8 years [http://www.hesonline.nhs.uk].

The healthy oral cavity hosts numerous bacterial species, including abundant streptococci (Lazarevic *et al.*, 2009). Oral microbial communities, both the commensals that promote health and potential pathogens that contribute to illness, are regulated by host defence factors originating from the local epithelial cells and neutrophils (Devine & Cosseau, 2008), and functioning in saliva. Also important are the palatine tonsils, positioned at the oropharyngeal inlet, which provide systemic as well as localized protection to counter the cellular attachment and invasion of microbes. The innate defences of the tonsils include a surface epithelium that functions not only as a protective barrier but also synthesizes an array of innate defence peptide molecules with antimicrobial and immunomodulatory activities (Agerberth & Gudmundsson, 2006; Bowdish *et al.*, 2006). These peptides include the cysteine-rich β-defensins, and the cathelicidin hCAP-18, the precursor protein of LL-37, a peptide shown by transgenic and knockout mouse models to have potent killing activity against group A streptococcus (Di Nardo *et al.*, 2008).

Evidence from work in the gastrointestinal tract also supports a role for host defence peptides, such as the defensins, in regulating the composition of commensal microbiota (Salzman *et al.*, 2010), which in turn contributes to general well-being and health. Studies of patients with recurrent inflammatory bowel disease (Crohn's) have shown that a reduced expression of defensins and the decreased antimicrobial activity of the gut are key pathogenic factors in the aetiology of the disease (Koslowski *et al.*, 2010; Wehkamp *et al.*, 2006).

The expression of the defensin and cathelicidin genes has been reported in tonsil samples from patients suffering recurrent acute tonsillitis (RAT), but their potential roles in the aetiology of the disease is less clear. Studies comparing the host defence peptides (HDP) expression profiles of tonsils from RAT patients and normal non-infected controls have yielded conflicting reports with expression being either elevated (Song *et al.*, 2006), or unchanged (Claeys *et al.*, 2003; Meyer *et al.*, 2006). These anomalies are unexplained but could reflect the small patient cohorts or the different ages of the patients in each cohort studied.

To investigate this further and the potential roles of the peptides in the susceptibility to recurrent tonsillitis, the messenger RNA (mRNA) expression profiles of 54 tonsil samples excised from control and RAT patients at the time of surgery were analysed and compared. Surgery to remove the tonsils of patients suffering from RAT was performed during the quiescent phase of the patient's disease and did not strictly reflect the innate immune responses of the tonsil tissues during an active streptococcal infection. To investigate this further, HaCaT cells, used as a model for the tonsil epithelium, and tonsil explants of control and RAT patients were challenged *in vitro* with the M1 *S. pyogenes* strain SF370, a strain shown previously to infect tonsil explants (Abbot *et al.*, 2007), and the effects of the infection on HDP expression were analysed.

METHODS

Bacterial strains

The serotype M1 *S. pyogenes* strain SF370, mutants thereof and the bacterial experimental growth conditions are as described previously (Abbot *et al.*, 2007).

Primary human tonsil tissue, challenge and adhesion assays

Palatine tonsils were obtained from patients undergoing tonsillectomy at the Freeman Hospital, Newcastle upon Tyne; they were used with informed consent and in compliance with Local Research Ethics Committee rules. Tonsils were excised for conditions including recurrent acute tonsillitis, obstructive sleep apnoea, enlarged tonsils and tonsillar crypts, and snoring. Tissues from patients with exceptionally scarred tonsils or from those who had undergone trauma during surgery, and in which the epithelia could not be differentiated from the lymphoid interior, were not used. Tonsils collected for RNA extraction were dissected immediately after excision. Whole tonsil dissection involved the intact epithelial surface being separated from the lymphoid interior of the tonsils and the samples being either snap frozen in liquid nitrogen or preserved in RNA Later (Ambion, Life Technologies, Grand Island, NY, USA).

For the *ex vivo* challenge experiments the tonsils were transported to the laboratory on ice, dissected and the infections were performed as described previously (Abbot *et al.*, 2007).

Immortalized cell culture

The HaCaT cells were sourced as described previously (Abbot *et al.*, 2007). Unless specified, media and supplements were purchased from Sigma-Aldrich (Poole, UK). HaCaT cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% (volume/volume) fetal calf serum, penicillin (100 U ml^{-1}) and streptomycin (100 $\mu g\ ml^{-1}$), and 110 $\mu g\ ml^{-1}$ sodium pyruvate. For the bacterial infection experiments HaCaT cells were seeded at a density of 1×10^6 cells per ml onto 14-mm diameter glass coverslips in 12-well tissue culture plates and incubated at 37°C and 5% CO_2 until confluent. Monolayers were washed three times with phosphate-buffered saline (PBS; Sigma) and incubated for 3 h in the appropriate medium lacking antibiotics, before adding bacteria.

RNA extraction and molecular analyses

Extraction of RNA from either cultured cells or the human tonsil samples was performed using the 'Pure Link Micro-to-Midi Total RNA Purification System' (Invitrogen, Life Technologies, Grand Island, NY, USA).

Quantitative reverse transcription–polymerase chain reaction

All RNA samples were pretreated, before reverse transcription (RT), with DNase (Promega, Southampton, UK) at a concentration of 1 U μg^{-1} RNA. The RT was performed at 42°C for 1 h, followed by heat inactivation for 5 min at 95°C. For human *β-defensin 1 (HBD-1)* and *hCAP-18/LL-37* gene amplification and quantification a Lightcycler® 480 probes master mix (Roche, Welwyn Garden City, UK) was used whereas *HBD-2, liver expressed antimicrobial peptide-2* (*LEAP-2*) and *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* analyses employed a Sybr® green master mix (Roche). Primers and annealing temperatures are as follows: HBD-1 forward: GTCAGCT CAGCCTCCAAAGGA, reverse: cgccGGTAGGAGGTTCTCATG GCG, (temp. (T) 60°C); hCAP18/LL-37 forward: cgctGACGGGC TGGTGAAGcg, reverse: CCCAGCAGGGCAAATCT CTT (temp. (T) 60°C); HBD-2 forward: GTGAAGCT CCCAGCCATCAG, reverse: GATTGCGTATCTTTG GACACC (temp. (T) 58°C); LEAP-2 forward: TGTT GGGCCAGATAGATG GCTC, reverse: GTTCCGA TACGTCTTTTTCTGCG (temp. (T) 58°C); GAPDH forward: CGGAGTCAAC GGATTTGGTC, reverse: CCG TTCTCAGCCTTGA (temp. (T) 58°C). Amplification conditions included a pre-incubation step of 2 min at 50°C, 2 min at 95°C and 45 cycles of 5 s at 94°C, 10 s at T°C, 10 s at 72°C. Samples without RT were included as negative controls. The mRNA transcripts were quantified in relation to a standard curve derived from dilutions of a cloned copy of the gene. Reactions were carried out in 96-well plates using a Lightcycler® 480 (Roche). All data were normalized to GAPDH.

Immunocytochemistry

Frozen human tonsil sections were fixed in 2% paraformaldehyde (PFA; Sigma) in PBS for 30 min

on ice, permeabilized with 0.1% Triton X-100 for 30 min at room temperature, blocked in 10% goat serum in PBS (Sigma) for 1 h on ice before incubation with anti-human LEAP-2 purified IgG (1 : 100 in PBS) overnight at 4°C. After incubation with goat anti-rabbit antibody-FITC (Chemicon, Millipore, Billerica, MA, USA), diluted 1 : 50 in PBS for 3 h the sections were mounted on slides with Vectashield. Sections were imaged by confocal laser scanning microscopy (TCS-NT Leica).

Time–kill antimicrobial assays

Time–kill assay methodology was adapted from previous (Townes *et al.*, 2009). Log phase cultures of *S. pyogenes* diluted to 5×10^5 in 90 μl PBS were incubated with recombinant BD1 (1 μg) (PeptaNova, Sandhausen, Germany), ±2 mM dithiothreitol (Schroeder & Wu, 2011) or PBS for 3 h at 37°C, sequentially diluted and each dilution plated onto Luria–Bertoni blood agar. All plates were incubated overnight at 37°C, and colonies were counted. Anti-microbial data were normalized to the PBS control to standardize bacterial numbers between assays.

Statistics

The *t*-tests and analyses of variance were performed and suitable post-tests were applied when appropriate. Dunnett's post-tests were applied when treatments compared with control were being considered. Bonferroni post-tests were used when more than one group of data was being compared.

RESULTS

HDP profiles of RAT and control patient tonsils

To establish whether alterations in tonsil HDP expression were linked to a patient's susceptibility to RAT the gene expression profiles of *HBD1* and *HBD2, cathelicidin/LL-37* and *LEAP-2* were determined in both the epithelial and lymph tissues of tonsils excised from control ($n = 21$) and RAT ($n = 33$) patients. Controls were undergoing treatment of obstructive sleep apnoea, enlarged tonsils and snoring, and not apparently associated with recurrent tonsillitis. Regardless of the HDP gene analysed, the expression profiles of the control and RAT cohorts were characterized by their variability, and when compared statistically, no significant differences in the expression of *HBD1, HBD2* or *cathelicidin/LL-37* were detected between the groups in either the epithelium or the lymphoid tissue (Fig. 1A–D). HBD1 expression was detected in the lymph samples of a number of the RAT patients, consistent with previous observations (Schwaab *et al.*, 2010) although these results may have reflected the contamination of such samples with epithelial material.

The LEAP-2 gene expression in the tonsil had previously been associated with four transcripts (Ball *et al.*, 2007), and LEAP-2 immunoreactivity was detected in both epithelial cells and lymphoid tissues (Fig. 1E). In this study the expression of the transcript encoding the secreted peptide, with antimicrobial activity, including against streptococci, was analysed (Howard *et al.*, 2010). As previously observed for other HDP genes the control and RAT tonsil cohorts were characterized by their intra-group variability and similarly no statistical differences in the mean LEAP-2 mRNA expression values were detected between the groups (Fig. 1D).

Children frequently present with RAT and within these patient cohorts was a group of 29 children aged < 10 years old. To identify whether the HDP expression profiles of this group were specifically impaired, their RAT tonsil HDP expression data were compared with that of age-matched controls. The age range was 3–9 years, and the median ages of the control and RAT groups were 4 years 6 months and 5 years, respectively. The tonsil HDP expression data relating to the children was again, regardless of the gene analysed, characterized by marked intra-group variability. Comparison of the mean hCAP-18/LL-37 mRNA expression data from the control and RAT epithelial samples (22 ± 11 compared with 8 ± 2 AU, respectively) was indicative of a reduction in expression in the RAT tonsils but was not significant, the data were skewed by two of the control tonsil values (127 and 63 AU, respectively) that were three to six times higher than the mean value (Fig. 2A). Similarly, no significant differences were observed between the mean LEAP-2, HBD1 or HBD2 mRNA expression values of the two groups (Fig. 2B–D). These data again indicated that regardless of age, recurrent tonsil infections were not linked to specific alterations in the tonsil HDP defences.

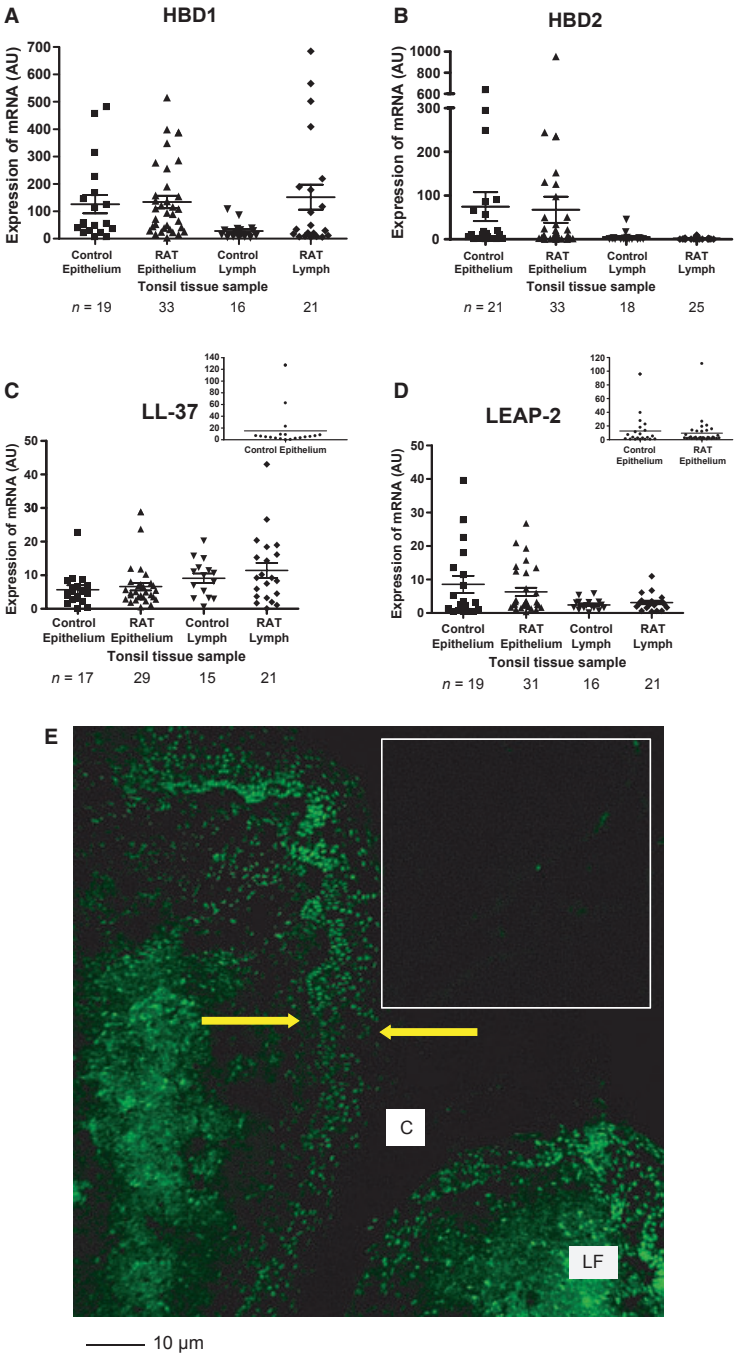

Figure 1 Expression of (A) human β-defensin 1 (HBD1), (B) HBD2, (C) hCAP-18/LL-37 and (D) liver expressed antimicrobial peptide-2 (LEAP-2) in tonsils removed from control and recurrent acute tonsillitis (RAT) patients with data normalized to GAPDH, presented in arbitrary units (AU) and according to tissue type and disease state. Individual values are presented (graphs inset to (C) and (D) include outliers), bars show mean values ± SEM; *n* = number of tonsil samples analysed in each group. (E) Immunohistochemical localization of LEAP-2 in tonsil removed from a RAT patient. LEAP-2 peptide detected with anti-human LEAP-2 purified IgG diluted 1 : 100 in phosphate-buffered saline and goat anti-rabbit antibody-fluorescein isothiocyanate diluted 1 : 50. C, crypt; LF, lymphoid follicle; arrows indicate epithelial layer. Insert shows staining of consecutive tonsil section without primary antibody.

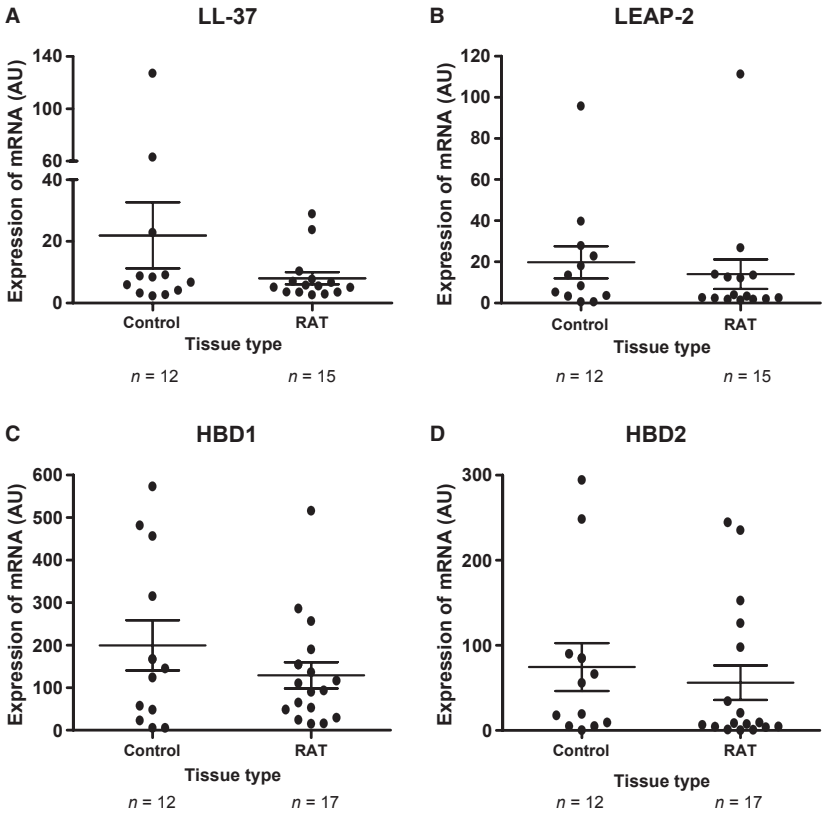

Figure 2 Expression of (A) hCAP-18/LL-37, (B) liver expressed antimicrobial peptide-2 (LEAP-2), (C) human β-defensin 1 (HBD1) and (D) HBD2 in tonsil epithelia from control and recurrent acute tonsillitis (RAT) individuals < 10 years of age. Data normalized to GAPDH, presented in arbitrary units (AU) and individual values presented; bars show mean values ± SEM; *n* = number of tonsil samples analysed in each group.

Streptococcal challenge of HaCaT cells and HDP responses

Host epithelia can respond to infection by up-regulating AMP gene expression (Zilbauer *et al.*, 2005; Ji *et al.*, 2009), but as the RAT patients recruited into this study were operated on between attacks of tonsillitis, and were not clinically infected with *S. pyogenes*, it could be argued that the observed tonsil HDP profiles reflected the quiescent state. To study an active infection, an *in vitro* HaCaT cell system, shown to provide an appropriate model for the tonsil epithelium (Abbot *et al.*, 2007), was adopted and the direct effects of *S. pyogenes* on HaCaT HDP gene expression was investigated following streptococcal challenges of up to 6 h. Bacterial binding to the cells was detected within 2 h of infection and increased up to 6 h (Fig. 3). At 4–6 h after infection the expression of *HBD1* and *HBD2* appeared reduced compared with the control values but only *HBD1* reached statistical significance ($P < 0.05$) (Fig. 3A, B). Conversely the trend was towards increased *LEAP-2* gene expression following the streptococcal challenge with this rise being significant ($P < 0.01$) at 6 h (Fig. 3C). As previously observed (Schauber *et al.*, 2006), *hCAP-18/LL-37* gene expression was low in the HaCaT cells, and following bacterial infection no significant changes in expression were detected (Fig. 4). Also, as reported previously (Schauber *et al.*, 2006). previous treatment of the cells with vitamin D enhanced *LL-37* gene expression and this upregulation occurred even in the presence of *Streptococci* (Fig. 4).

It has been shown previously that pili, expressed on the surface of *S. pyogenes*, affect the *in vitro* infectivity of the bacterium as the deletion of genes encoding the pilus-like structures prevents bacterial adhesion to host cells (Abbot *et al.*, 2007). To determine the absence of pili, and binding, on the HDP responses of the HaCaT cells to *Streptococci*, the

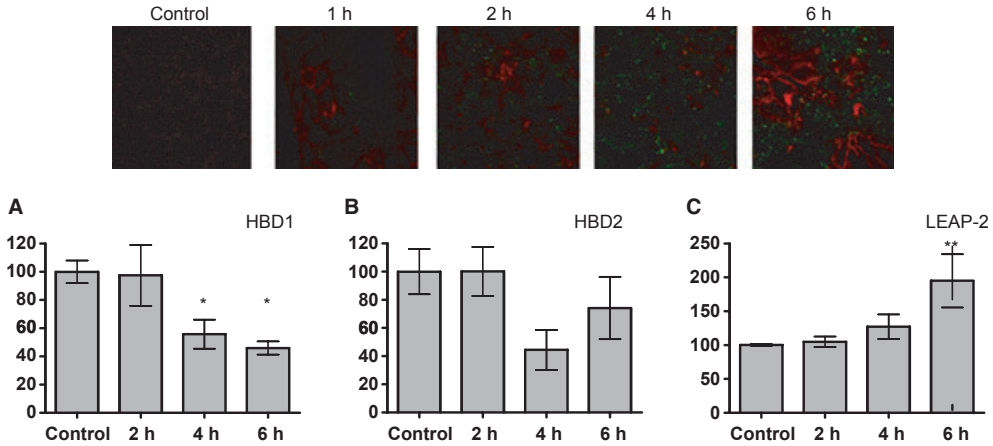

Figure 3 Bacterial adhesion of HaCaT cells at 1, 2, 4 and 6 h of infection with wild-type *Streptococcus pyogenes* engineered to express green fluorescent protein plasmid (green). HaCaT cells have been stained with Phalloidin-TRITC (red). Expression of (A) human β-defensin 1 (HBD1), (B) HBD2 and (C) liver expressed antimicrobial peptide-2 (LEAP-2) mRNA in HaCaT cells infected for 2, 4 and 6 h with *S. pyogenes* M1 serotype (wild-type). Data normalized to GAPDH and expressed as a percentage of control. Values represent mean ± SEM and each value is a mean of a minimum of three independent experiments and five to eight replicates. *$P < 0.05$ compared with control; **$P < 0.01$ compared with control.

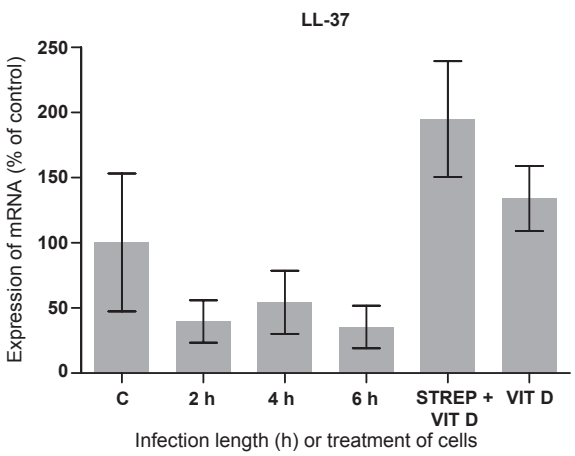

Figure 4 Expression of hCAP-18/LL-37 mRNA in HaCaT cells infected for 2, 4 and 6 h with *S. pyogenes* M1 serotype (wild-type) and in conjunction with vitamin D treatment. Data normalized to GAPDH and expressed as a percentage of control. Values represent mean ± SEM and each value is a mean of two experiments and three replicates.

M1 mutant strain *Δspy0129* that lacks the pilus-associated Sortase C (SrtC1) and fails to synthesize pili (Abbot *et al.*, 2007), and strain *Δspy1154* that lacks Sortase A that catalyses the linkage of proteins, and protein complexes such as pili, to the cell wall (Barnett & Scott, 2002; Race *et al.*, 2009), were used to infect the HaCaT cells. Bacterial binding (Fig. 5), indicated that the *S. pyogenes* pili-defective mutants, unlike the wild-type streptococci, did not bind to the HaCaT cells. Expression data for HDP at 6 h of bacterial challenge again revealed HBD1 expression to be significantly reduced ($P < 0.01$), following infection with either wild-type or the pili-defective mutants (Fig. 5A). The LEAP-2 data in contrast were not as definitive (Fig. 5B); cells challenged with wild-type and strain *Δspy1154* supported enhanced LEAP-2 expression but those challenged with strain *Δspy129*, lacking the pilus-associated Sortase C, did not. These data suggested that the regulation of HDP expression by streptococci is complex but not dependent on pili and bacterial binding. Experiments in which HaCaT cells were challenged with bacterial cell wall components including lipopolysaccharide and lipoteichoic acid indicated that the reagents in isolation had no effects on HDP gene expression (data not shown).

Streptococcal challenge of tonsils and HDP responses

The HaCaT cell data indicated that *in vitro*, the *S. pyogenes* M1 serotype is able to modulate the gene expression patterns of the HDPs, suggesting a potential mechanism by which the bacterium, a causal agent in tonsillitis, can evade the tonsil innate response and cause disease. To authenticate these

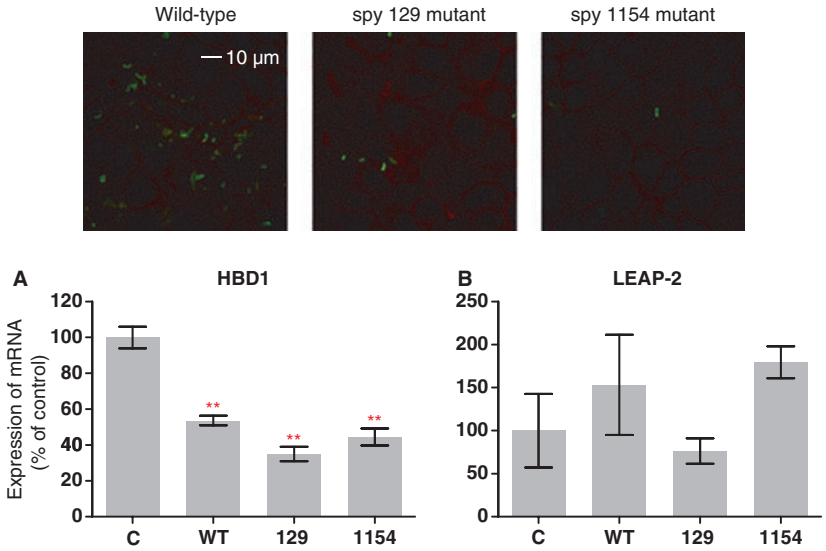

Figure 5 Bacterial adhesion to HaCaT cells after 6 h of infection with *Streptococcus pyogenes* M1 wild-type and pili-defective mutants *Δspy129* and *Δspy1154* expressing green fluorescent protein plasmid (green). HaCaT cells have been stained with Phalloidin-TRITC (red). Expression of (A) human β-defensin 1 (HBD1) and (B) liver expressed antimicrobial peptide-2 (LEAP-2) mRNA in HaCaT cells infected for 6 h with *S. pyogenes* M1 serotype (wild-type), and pili defective mutants *Δspy129* (129) and *Δspy1154* (1154). Data normalized to GAPDH and expressed as a percentage of control. Values represent mean ± SEM and each value is a mean of two experiments and three or four replicates. $^{**}P < 0.01$ compared with control.

data, tonsil explants prepared from freshly isolated samples of human palatine tonsil from control and RAT patients were maintained on filter supports, challenged for up to 6 h with *S. pyogenes* M1 wild-type and HDP gene expression patterns in response to the acute infection determined. As in the HaCaT cells the streptococci bound to the individual tonsil sections within 2 h of the challenge (Fig. 6, panels 1–4).

The tonsil HDP expression profiles determined following *Streptococcal* binding showed marked inter-sample variability within each group (Fig. 6A–C), similar to that observed in the clinical study (Fig. 1). Following infection, *hCAP-18/LL-37* expression in both the control and RAT tonsils showed no statistically significant changes although there was a trend for the transcript levels to be elevated at 2 h but reduced at 4 h after infection (Fig. 6A), the latter similar to that observed in the HaCaT cells. Contrary to what was observed in the HaCaT cells, *HBD1* gene expression was significantly increased ($P < 0.01$) in the control patient tonsil sections following 2 h of bacterial exposure (Fig. 6B), although by 4 h *HBD1* expression was comparable to control. This response was not observed in the RAT patient tonsils where no significant change was detected. Comparable to that observed in the HaCaT cells the HBD2 mRNA expression values were lower in the control patient tonsils following the 4 h streptococcal infection although no comparable trends were identified in the RAT patient tonsil sections. Data relating to the effects of the *S. pyogenes* M1 serotype infection on *LEAP-2* expression patterns revealed no statistically significant changes (data not shown).

HBD1 antimicrobial activity

Antimicrobial assays performed using HBD1 supported bacterial killing of *S. pyogenes* and inclusion of the reducing agent dithiothreitol indicated a 10% enhancement of bacterial killing compared with HBD1 alone (Fig. 7).

DISCUSSION

Despite RAT being relatively common, the physiological and immunological factors that facilitate *S. pyogenes* infection remain elusive. In humans, HDPs function as part of the innate defences and provide a primary host defence mechanism. A deficiency of HDPs has been associated with susceptibility to infection (Morrison *et al.*, 2002) and this study investigated

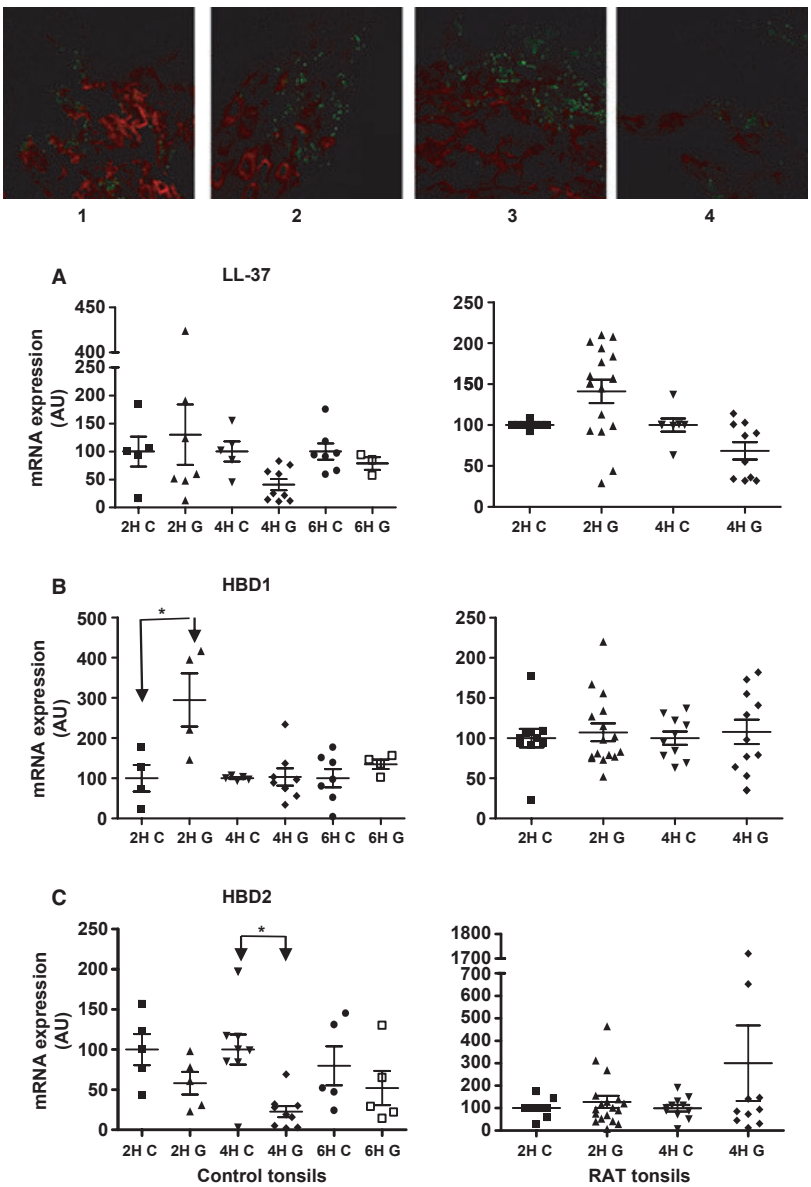

Figure 6 (1–4) Bacterial adhesion to tonsil explants (four different tonsils) after 2 h of infection with *Streptococcus pyogenes* M1 serotype wild-type expressing green fluorescent protein plasmid (green). Tonsil tissues have been stained with Cytokeratin-14 (red). Expression of hCAP-18/LL-37 (A), human β-defensin 1 (HBD1) (B), HBD2 (C) mRNA in tonsil explants incubated with *S. pyogenes* M1 serotype wild-type. Expression relates to tonsil sections challenged with either buffer (C) or group A streptococci (G) for 2, 4 or 6 h (H). Data normalized to GAPDH and expressed as a percentage of control. Values represent mean ± SEM. $^*P < 0.05$ compared with control. Each symbol represents an individual HDP expression value (tonsil explant numbers range from 3 to 18). Left hand side graphs show data relating to tonsils removed from patients not suffering RAT; right hand side graphs show data relating to infection of tonsils removed from recurrent acute tonsillitis (RAT) patients.

whether a deficiency in HDPs at the tonsil epithelial surface contributes to RAT. HDP expression analyses of 54 tonsil samples excised from control and RAT patients at the time of surgery did not support this. All the HDP genes examined were expressed in the tonsils of those suffering RAT and statistically the expression levels between the RAT and control groups were not different.

The RAT tonsils were excised from patients about to undergo a tonsillectomy and free from active infection (tonsillitis). Synthesis of HDPs is regulated in response to microbial and environmental stimuli, so it

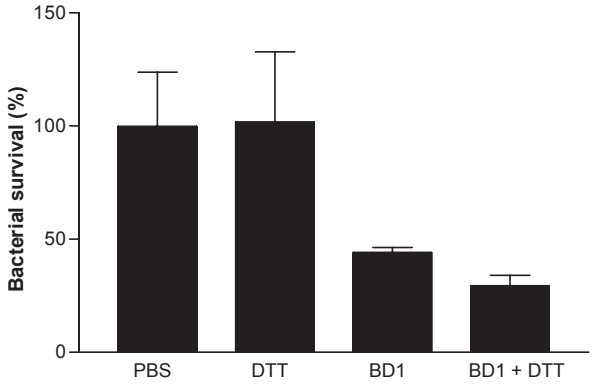

Figure 7 Human β-defensin 1 (HBD1) antimicrobial activity against *Streptococcus pyogenes* M1 serotype wild-type. The *S. pyogenes* M1 serotype wild-type was incubated with phosphate-buffered saline (PBS), dithiothreitol (DTT), 1 μg HBD1 or 1 μg HBD1 plus 2 mM DTT. Values represent mean ± SEM and each value is a mean of two experiments and five or six replicates.

was perhaps not surprising that during this quiescent phase no differences in HDP profiles were observed between the tonsils of the RAT and control patient groups. To explore this further HaCaT cells shown previously to model the tonsillar epithelium (Abbot *et al.*, 2007) were used and reductions in HDP gene expression, observed within hours of a streptococcal challenge and epithelial binding, suggested a potential mechanism by which the bacterium, a causal agent in tonsillitis, is able to evade the host innate response and breach the tonsil epithelial barrier. Downregulation of HDP gene expression is a mechanism by which pathogenic bacteria have been shown to suppress the immune system long enough to cause infection (Quinn & Cole, 2007). The use of bacterial mutants indicated that the alterations in HDP gene expression mediated by *S. pyogenes* were independent of bacterial binding although the identities of the streptococcal factors responsible for initiating the changes in mRNA expression were not identified. Enteric pathogens have been reported to suppress HDP expression through the activities of their virulence proteins (Chakraborty *et al.*, 2008) so the roles of *S. pyogenes* toxins including streptolysin S as transcriptional regulators cannot be excluded.

The HaCaT HDP expression data although allowing an insight into mechanisms by which *S. pyogenes* can evade the host tonsil innate defences still could not explain why some individuals are susceptible to *S. pyogenes* infection and others not. To address this further the *ex vivo* tonsil model was used because this allowed differences between the HDP expression profiles of tonsils removed from control and RAT patients in direct response to a *S. pyogenes* M1 challenge to be examined and compared.

The tonsil explant HDP expression profiles determined following streptococcal binding showed marked variability that related specifically to individual tonsil samples within each of the groups, and tended to mask any significant trends. The suggestion of an early increase in the expression of *hCAP18/LL-37* in both control and RAT patient tonsils was supportive of the induction and protective functions of the epithelial synthesized peptide in response to streptococcal infection, although by 4 h this response was diminished consistent with gene downregulation. Vitamin D deficiency has been reported in children undergoing adeno-tonsillectomy and the *hCAP18/LL-37* gene is known to respond to vitamin D treatment (Fig. 4) (Reid *et al.*, 2011; Schauber *et al.*, 2006). The fact that comparable trends in gene expression were detected in control and RAT patient tonsils following infection suggests that this HDP does not, by itself, play a major role in the susceptibility to RAT and as such the preventative and therapeutic potential of vitamin D in RAT treatment appears limited.

Host *HBD1* expression is regarded as constitutive, although inducible expression has been reported in oral keratinocytes following stimulation with phorbol 12-myristate 13-acetate (PMA), and bacteria associated with periodontal diseases including *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* (Joly *et al.*, 2005; Vankeerberghen *et al.*, 2005). Tonsils from control patients were characterized by increased HBD1 gene expression and within 2 h of a streptococcal challenge, an increase that was not mirrored in the RAT tonsils. Immunohistochemical data (Ball *et al.*, 2007) and HBD1 peptide concentrations measured in tonsils removed from subjects suffering from acute tonsillitis (Schwaab *et al.*, 2010) also suggest that HBD1 is reduced during tonsillitis, which supports HBD1 as being significant in the tonsillar epithelial defences (Prado-Montes de Oca, 2010). Compared with other defensins, HBD1 is classed as a weak antimicrobial agent (Bensch *et al.*, 1995; Pazgier *et al.*, 2007) although in anaerobic niches, such as those that exist in the tonsil, the internal di-sulphide bonds of HBD1 can be reduced, resulting in the production of linear HBD1 molecules that

have increased antimicrobial killing capacity against bacteria, including gram-positives (Schroeder & Wu, 2011). Our time–kill data supported HBD1 as have killing capacity against the M1 *S. pyogenes* strain SF370, and that killing was enhanced in a reducing environment. The susceptibility of the streptococci to HBD1 killing was in conflict with previous observations (Fernie-King *et al.*, 2004); our data may reflect the use of a different strain of the M1 serotype or the bacterial preparation method used resulting in the dilution or exclusion of potential streptococcal derived inhibitors of host defence peptides (Frick *et al.*, 2003). However, taken together, our data suggest that the rapid increase in tonsillar HBD1 and the potential reduction of the peptide through redox modulation is of significance in the innate defence of the tonsil epithelial barrier against *S. pyogenes* infection. Single nucleotide polymorphisms (SNPs) in the *DEFB1* gene promoter have been linked to susceptibility to inflammatory diseases including dental caries and periodontitis (Ozturk *et al.*, 2010; Schaefer *et al.*, 2010), but whether such polymorphisms are linked specifically to RAT patients is not known.

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