The Constitutive Capacity of Human Keratinocytes to Kill *Staphylococcus aureus* Is Dependent on β-Defensin 3

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Normal skin is often exposed to bacteria, including potent pathogens such as *E. coli*, *Staphylococcus aureus*, and *Streptococcus* sp., but these microbes usually do not cause skin inflammation or infection in healthy individuals. Therefore, we hypothesized that there must be a constitutive mechanism for rapid destruction and elimination of small numbers of bacteria which penetrate the stratum corneum from everyday activities. This study found that exposure of keratinocytes cultured from a number of individuals to *S. aureus* resulted in approximately 2–3 log better killing than by HaCaT cells within 1 hour. Killing required contact between the keratinocytes and the bacteria, but was not dependent on internalization. Contact between the bacteria and the keratinocytes resulted in rapid deposition of several antimicrobial peptides onto the bacteria, but only human β-defensin (HBD) 3 accumulated at levels sufficient to account for killing when *S. aureus* were exposed to human skin explants. Blocking peptide binding of HBD3 inhibited killing of the bacteria, indicating an essential role for β-defensin 3 in the constitutive killing of bacteria by normal keratinocytes.

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INTRODUCTION

The skin is exposed to bacteria, including potent pathogens almost continually. However, infection of normal healthy skin rarely occurs. *Staphylococcus aureus* is an important human pathogen, which can be isolated from the skin or nares of approximately 30% of the population (Kluytmans *et al*., 1997; Fridkin *et al*., 2005). *S. aureus* is a primary cause of skin and soft tissue infections (Rennie *et al*., 2003), necrotizing fasciitis (Miller *et al*., 2005), and sepsis (Biedenbach *et al*., 2004). Therefore, it is important to understand the innate mechanisms employed by the normal epidermis to prevent invasion and infection by bacteria in general, and *S. aureus* in particular.

The superficial cornified layer of the epidermis is an important aspect of the defensive barrier presented by the skin. However, this layer is frequently breached by small abrasions from everyday activities. This process undoubtedly introduces bacteria directly into the layers of viable keratinocytes, which make up the epidermis. It is well known that keratinocytes can sense the presence of bacteria via Toll-like receptors (Esche *et al*., 2004), which results in synthesis and secretion of inflammatory mediators, and the recruitment of antimicrobial effector cells such as macrophages and neutrophils (Mempel *et al*., 2003). These phagocytes are usually credited as the first line of defense against microbial invasion via the skin (Janeway *et al*., 2005).

However, in addition to Toll-like receptors, keratinocytes themselves bear some surface receptors in common with professional phagocytes, including Fc receptors (Tigalonowa *et al*., 1990; Bjerke *et al*., 1994), and complement receptors (Hunyadi *et al*., 1991; Dovezenski *et al*., 1992). In addition, keratinocytes can be induced to synthesize reactive nitrogen intermediates (RNIs), defensins, and cathelicidin (Kroll *et al*., 1999; Ali *et al*., 2001; Dorschner *et al*., 2001), which are potent microbicides. We have previously shown that keratinocytes in the epidermis contain sufficient inducible antimicrobial peptides (AMPs) to contribute to microbial killing (Ali *et al*., 2001). In addition, Braff *et al*. (2005) have shown that cathelicidin contributes partially to direct activity against cathelicidin-sensitive *S. aureus* mutants. Despite these observations, the biological significance of keratinocyte expression of AMPs in human skin is not completely worked out. We have developed an *ex vivo* model to examine the interaction and elimination of *S. aureus* by keratinocytes in

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Abbreviations: AMP, antimicrobial peptide; DAPI, 4′,6-diamidino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBD, human β-defensin; KGM, keratinocyte growth medium; l-NAME, N-nitro-L-arginine methyl ester; NJMRC, National Jewish Medical and Research Center; PBS, phosphate-buffered saline; RNI, reactive nitrogen intermediate

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human skin explants, and shown that these cells possess a novel constitutive mechanism to kill *S. aureus* within minutes of contact between the bacteria and the cell surface. The mechanism of this killing depends on deposition of human β-defensin (HBD)-2 and -3 onto the surface-bound bacteria. These studies highlight the essential role that keratinocytes play in deployment of AMPs to rapidly kill *S. aureus*, which penetrate the stratum corneum.

RESULTS

During the course of examining biopsies of human skin exposed to Texas Red-labeled *S. aureus*, we observed that the keratinocytes in the viable epidermal layers which were exposed to the bacteria rapidly bound the bacteria, and accumulated Texas Red-labeled debris derived from the bacteria. Figure 1 represents the pattern of binding and accumulation observed from 12 donors. Some of the cells in the keratinocyte layers contained what appeared to be accumulations of cell envelope material in the cytoplasm. We hypothesized that the label initially became associated with the cells as intact bacteria, and was then rapidly digested and internalized by keratinocytes. As internalization was mainly observed near the edges of the biopsy, and toward the middle of the epidermal layer, we hypothesized that viable keratinocytes, rather than non-viable cell remnants of the cornified layers are most active in this regard. In order to test this hypothesis, we placed primary human keratinocytes into culture, and allowed them to differentiate in the presence of Ca^{2+} for 5 days. When *S. aureus* were labeled with Texas Red and added to the cultured human keratinocytes, they became associated with the cells within 5 minutes (Figure 2a). After 15 minutes, the keratinocytes had internalized several bacteria per cell, and most of the red fluorescence was associated with recognizable bacteria within the cytoplasm (Figure 2b). Internalization rather than simple surface association is supported by three-dimensional microscopy, clearly showing the plasma membrane surrounding the bacteria in all three view axes (Figure S1). Examination of the cultures after 1 hour, however, revealed that the majority of the red fluorescence was no longer associated with recognizable bacteria, suggesting that the Texas Red label and the cellular macromolecules to which they were covalently attached had been removed from the bacteria (Figure 2c). Therefore, we hypothesized that the bacteria were destroyed by the cells. Although the cells shown in Figure 2a–c were quite active in accumulation of *S. aureus*, an overview of the cultures at 1 hour revealed that only certain groups of cells in the culture actively internalized *S. aureus*, whereas other cells were completely inactive in this regard (Figure 2d). This apparent destruction of *S. aureus* by human keratinocytes has not been previously reported, so we sought to test directly whether contact between the keratinocytes and the bacteria resulted in loss of bacteria viability.

In order to determine the capacity of keratinocytes to kill *S. aureus*, it was necessary to use a technique which could quantitate the total number of organisms which came into

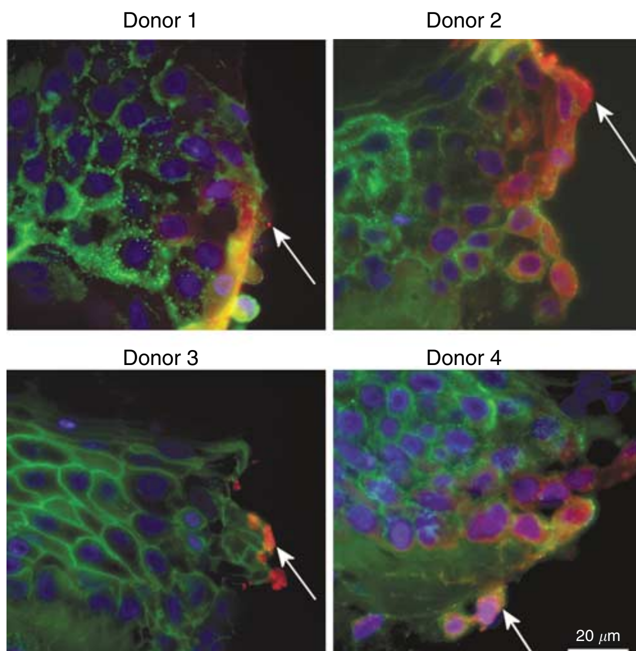

Figure 1. Accumulation of *S. aureus* debris by keratinocytes in human skin. Four representative biopsies ($n=12$) of human skin exposed to Texas Red-labeled *S. aureus* (Wood 46) (red, arrow) for 30 minutes. After fixation and mounting, the tissues were counterstained with FITC wheat germ agglutinin (green) and DAPI (Blue). The images are single confocal planes through the samples. Arrows highlight *S. aureus* particles, which appear to still contain DNA. Bar = 20 μm.

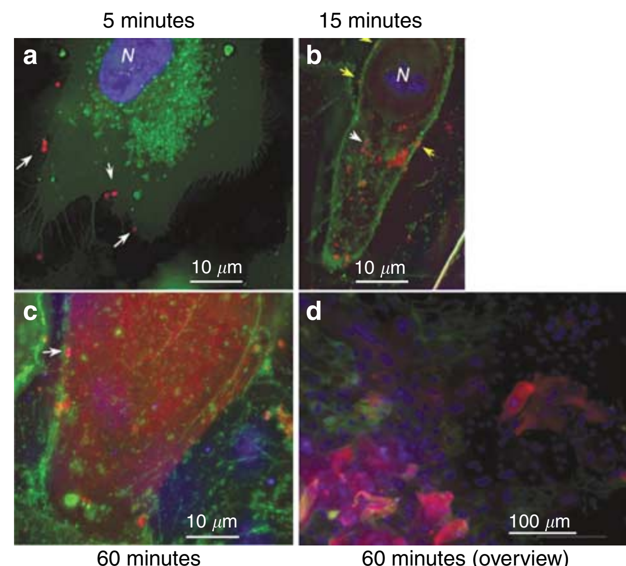

Figure 2. Accumulation of *S. aureus* (Wood 46) and destruction by cultured human keratinocytes. (**a**) A single confocal plane through a representative (three experiments) keratinocyte (wheat germ agglutinin – Oregon Green, green) exposed to 1×10^6 serum opsonized *S. aureus* (Wood 46)/ml for 5 minutes has bound bacteria (arrows) (Texas Red, red). (**b**) After 15 minutes, keratinocytes have internalized multiple bacteria (arrows). (**c**) After 1 hour, keratinocytes have accumulated substantial amounts of Texas Red-labeled material derived from *S. aureus*. (**d**) Low magnification view of the culture, showing clusters of cells, which accumulate *S. aureus*. Cell nuclei (N) and bacterial DNA are presented in blue (DAPI). Bar = 10 μm.

contact with the keratinocytes, and those which remained viable over time. This was accomplished by labeling the bacteria with ^{3}H uracil, and determining the amount of radioactivity associated with a single colony-forming unit at the beginning of the experiment. Table 1 shows that the bacteria grew very little in keratinocyte growth medium (KGM), yet retained the ^{3}H uracil label. Figure 3a is representative of three experiments, and shows the results of exposing ^{3}H-labeled *S. aureus* to either a keratinocyte-like cell line, HaCaT (Scalettar *et al.*, 1996), or to primary human keratinocytes over 5 hours. After only 1 hour of exposure to primary keratinocytes, they killed 2–3 log more bacteria than HaCaT cells. This suggested that normal human keratinocytes express a mechanism for rapidly killing *S. aureus*, which come into contact with them. This was not an artifact of lysing the cells to recover the bacteria, because bacteria exposed to HaCaT cells were processed in the same manner, yet remained viable. It was also not due to residual antibiotics in the medium, as washing out the antibiotics 48 hours before the experiment yielded the same results as washing them out 24 hours before the experiment (Figure S2). Contact between the keratinocytes and the bacteria was required for killing, as conditioned medium from *S. aureus*-stimulated cultures had no effect on the viability of *S. aureus* when exposed for the same duration (data not shown). In a similar experiment, labeled *S. aureus* were exposed to human peripheral blood neutrophils. The neutrophils were able to accumulate, and kill *S. aureus* to a greater degree, than the keratinocytes or the HaCaT cells, consistent with their role as professional microbicidal phagocytes (Janeway *et al.*, 2005).

As keratinocytes express Fc and complement receptors commonly associated with phagocytes (Tigalonowa *et al.*, 1990), and have been shown to endocytose different types of particles, we hypothesized that primary human keratinocytes might utilize internalization as part of the killing mechanism, similar or identical to the killing mechanism of neutrophils and macrophages.

There are two processes by which particles as large as *S. aureus* might be internalized by keratinocytes: macropinocytosis and phagocytosis. We next sought to determine the role of each process during killing and internalization of *S. aureus*. Macropinocytosis and phagocytosis can be specifically inhibited by amiloride and colchicine, respectively (Boukamp *et al.*, 1988; West *et al.*, 1989). Therefore, these pharmacologic agents provide tools to determine the contribution of each of these internalization pathways to uptake of *S. aureus* by keratinocytes. Figure 4 is representative of three experiments, and shows that both colchicine (Figure 4a) and amiloride (Figure 4b) inhibit internalization of *S. aureus* by keratinocytes in a dose-dependent manner. This confirms the above results obtained by three-dimensional microscopy (Figure S1). These results also indicate that the internalization of the bacteria is an active cellular process, rather than invasion of the cells by the bacteria. A maximum of approximately 17% of serum-opsonized *S. aureus*, and 10% of non-opsonized, cell-associated bacteria were internalized (i.e., resistant to gentamicin) in the absence of any inhibitor. Colchicine inhibited internalization of serum opsonized viable *S. aureus*, as determined by lysis of the cultures and plating, by approximately 66% when 33 μM was added to the medium before addition of the opsonized

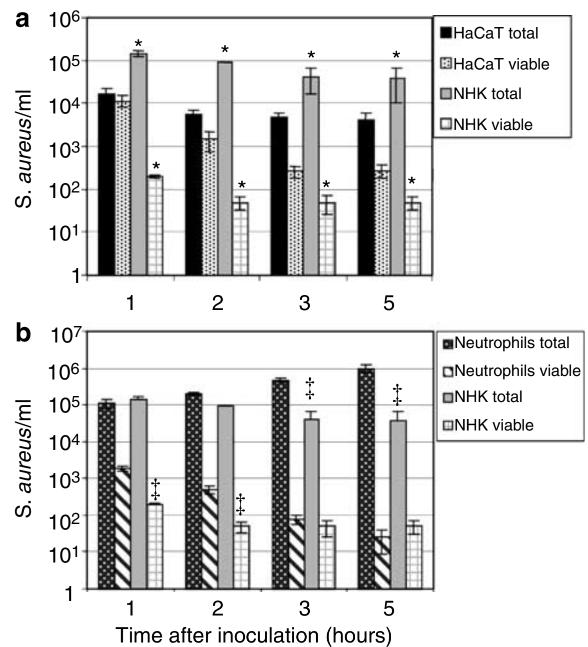

Figure 3. Comparison of microbicidal activity of HaCaT *versus* normal human keratinocytes. HaCaT and normal human keratinocytes were incubated with *S. aureus* (ATCC29213) for 1 hour. After washing, the monolayers were lysed and total bacteria determined radiometrically, whereas viable bacteria were determined by colony-forming unit. The number of total and viable bacteria is shown on the *y* axis as a function of time co-culture. (**a**) Primary human keratinocytes compared with HaCaT cells. (**b**) Primary human keratinocytes compared with primary human neutrophils. **P*<0.01 relative to HaCaT at the same time point. ‡*P*<0.01 relative to neutrophils at the same time point. Results are representative of three experiments. Error bars represent standard deviation of triplicate samples.

Table 1. Growth of *S. aureus* and retention of ^{3}H uracil in KGM

Time after inoculation	0	1	2	3	5
CPM	6.3×10^6	6.2×10^6	6.0×10^6	6.3×10^6	6.2×10^6
CFU	2.24×10^5	2.38×10^5	2.17×10^5	2.52×10^5	2.88×10^5
CPM/CFU	28.1	26.1	27.6	25.0	21.5

CFU, colony-forming unit; CPM, counts per minute; KGM, keratinocyte growth medium; *S. aureus*, *Staphylococcus aureus*.

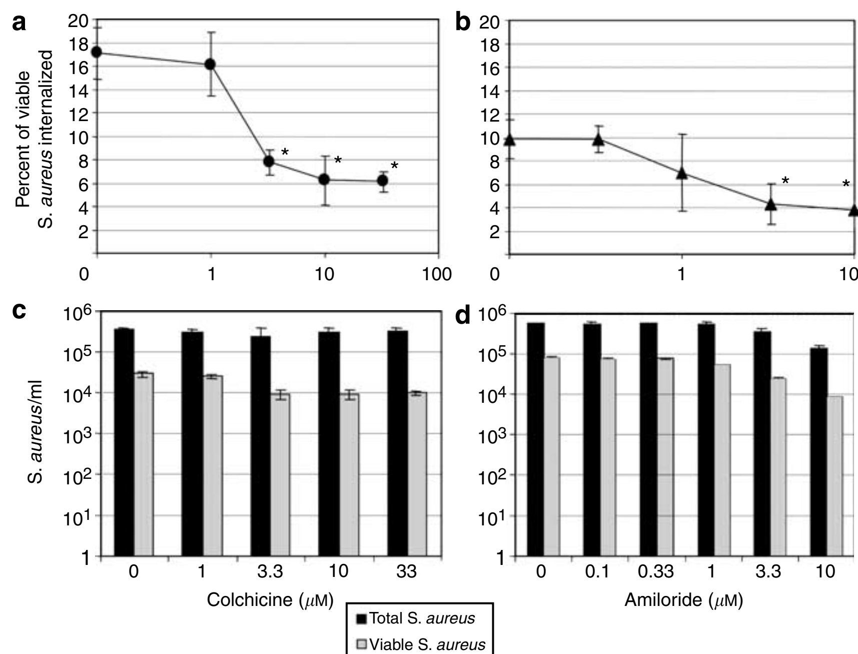

Figure 4. Pharmacologic inhibition of internalization. (**a**) Percentage of viable, serum opsonized *S. aureus* (ATCC29213) recovered from lysates of normal human keratinocytes (NHK) cells after 30 minutes pretreatment with colchicines, and killing of extracellular *S. aureus* with gentamicin. (**b**) Recovery of viable non-opsonized *S. aureus* from NHK after 30 minutes preincubation with amiloride at the concentrations shown on the *x* axis, and killing of extracellular *S. aureus* with gentamicin. (**c**) Ability of keratinocytes to kill *S. aureus* with increasing concentrations of colchicine, presented as total *versus* viable bacteria. (**d**) Ability of keratinocytes to kill *S. aureus* in the presence of amiloride, presented as total *versus* viable bacteria. Error bars represent standard deviation of triplicate samples. Results are representative of three experiments. *$P<0.05$ relative to no inhibitor.

bacteria (Figure 4a). This concentration was sufficient to block <95% of horseradish peroxidase uptake in preliminary experiments (data not shown). Increasing concentrations of amiloride were able to inhibit internalization of cell-associated, viable *S. aureus*, which had not been opsonized, with maximum inhibition occurring at the highest concentration of 10 mM. This indicates that in the absence of opsonization, the internalization pathway responsible for most of the internalization is amiloride sensitive, and in that respect similar to macropinocytosis. Therefore, internalization of *S. aureus* by keratinocytes can occur via pathways dependent on both phagocytosis and macropinocytosis. However, although inhibition of macropinocytosis and phagocytosis, inhibited internalization, neither inhibitor blocked killing of the bacteria by the cells as shown in Figure 4c and d. Furthermore, neither chemical had any effect on the growth rate of the bacteria (data not shown).

Therefore, killing of *S. aureus* appears to be independent of internalization, suggesting that it may occur soon after contact between the bacteria and the cell surface. Indeed, Figure 4c and d suggested that keeping the bacteria at the surface of the cells by inhibiting internalization actually enhances killing. Understanding the microbicidal mechanism, which occurs after binding or during internalization, resulting in loss of bacterial viability is important. In order to accomplish this, we examined *S. aureus* in association with keratinocytes for evidence of exposure to the most likely antimicrobial mechanisms: reactive nitrogen production and release of AMP.

Assessment of keratinocyte cultures for production of RNIs showed that RNIs were indeed generated in response to *S. aureus*, but only became detectable after 24 hours of exposure (data not shown). Inclusion of increasing amounts of *N*-nitro-L-arginine methyl ester (l-NAME) in the killing assay to inhibit any production of RNI did not inhibit the ability of keratinocytes to kill *S. aureus* (Figure 5). Therefore, we concluded that the rapid bactericidal activity of keratinocytes was not dependent on production of RNI.

Additional keratinocyte products, which may be important for the microbicidal activity are AMP, including LL37, HBD1, HBD2, and HBD3. We have measured the sensitivity of *S. aureus* to these AMP both in low salt medium (0.01 × tryptic soy broth, 10 mM NaPOh, pH 7.2), and the KGM, which contains physiological levels of salt. Table 2 illustrates the concentrations of each AMP, which killed 99.9% of *S. aureus* in 2 hours, and standard deviation of at least three independent experiments for each peptide (defined as the bactericidal concentration, $BC_{99.9}$). In KGM, HBD3 was the most potent of the four AMP tested ($BC_{99.9}=10.70\pm5.75$ μM), followed by LL37 (43.68 ± 18.39 μM) and HBD2 (483.03 ± 43.22 μM). We were unable to detect any microbicidal activity of HBD1 for *S. aureus*.

Next, we sought to determine how much of each AMP the *S. aureus* were exposed to when in contact with the keratinocytes. In order to accomplish this, we exposed *S. aureus* in KGM to increasing concentrations of each peptide, rapidly rinsed them by centrifugation, and then fixed the bacteria with paraformaldehyde. Simultaneously, *S. aureus*

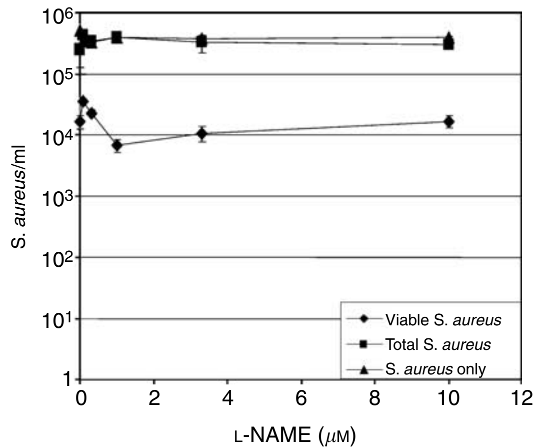

Figure 5. Effect of inhibitors of reactive nitrogen generation. Keratinocytes treated with increasing concentrations of l-NAME to inhibit generation of RNI were exposed to *S. aureus* (ATCC 29213). The number of total (squares) and viable cell-associated bacteria (diamonds). Bacteria exposed to l-NAME in KGM in the absence of cells are shown by triangles. Error bars (in some cases smaller than the plot symbols) represent standard deviation of triplicate samples. Results are representative of three experiments.

Table 2. $BC_{99.9}$ values of AMP in 0.01 × TSB and KGM for *S. aureus*[1]

Peptide	0.01 × TSB, 10 μM NaPOH	KGM
LL37	2.3±0.54 μM	43.68±18.39 μM[1]
HBD1	>1,000 μM	>1,000 μM
HBD2	432.99±30.75 μM	483.03±43.22 μM
HBD3	4.64±4.08 μM	10.70±5.75 μM[1]

AMP, antimicrobial peptide; $BC_{99.9}$, bactericidal concentration 99.9; KGM, keratinocyte growth medium; TSB, tryptic soy broth; *S. aureus*, *Staphylococcus aureus*.
[1]$P<0.05$ by *t*-test, relative to the $BC_{99.9}$ of the same peptide in 0.01 × TSB, 10 μM NaPOH.

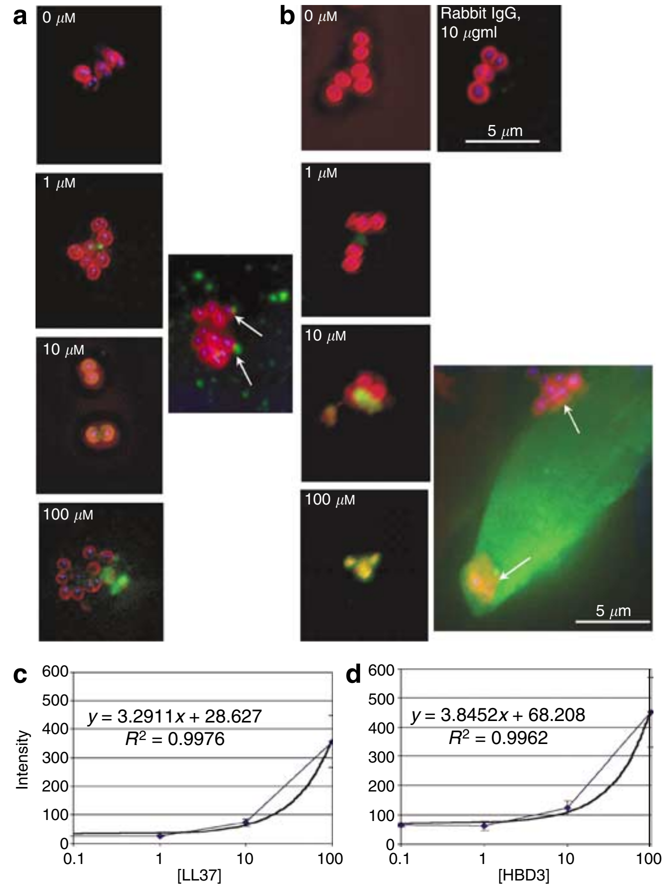

Figure 6. Intensity of AMP staining on *S. aureus* (Wood 46) after exposure to pure peptide or keratinocytes. Single confocal images of *S. aureus* (Wood 46) labeled with Texas Red (red) were exposed to increasing concentrations of LL37 (left column), or HBD3 (right column), and stained with antibodies specific for those peptides. The rabbit isotype control is shown to the right. In parallel, bacteria were exposed to keratinocyte cultures for 1 hour, fixed, and stained with the same antibodies. Representative bacteria in association with keratinocytes stained for (**a**) LL37 or (**b**) HBD3 are placed near the standard image of similar intensity. Bar = 5 μm. The intensity of each exposure series is plotted against the concentration of (**c**) LL37 or (**d**) HBD3. Regression analysis, line equations, and correlation coefficients are shown on each graph.

were exposed to cultures of differentiated keratinocytes for 1 hour, rinsed, and fixed with paraformaldehyde. Both the bacteria and the cell cultures were then stained using immunofluorescent reagents specific for the AMP in question as shown in Figure 6. The AMP fluorescence intensity of the bacteria exposed to the pure peptides was then compared with the intensity of the bacteria in contact with the keratinocytes. AMP exposure of the *S. aureus* in contact with keratinocytes was then estimated based on the curve of fluorescence intensity *versus* concentration of AMP. The results, which are representative of three experiments, shown in Figure 6c and d reveal how the fluorescence intensity of antibody staining of the AMP increased with increasing exposure of the bacteria to purified AMP. Images of *S. aureus* in contact with keratinocytes (Figure 6a and b) are placed near the standard image with most similar intensity. Regression analysis of intensities derived from at least 20 bacteria from each of triplicate samples ($N=60$) revealed that the bacteria were exposed to 34.2 μM HBD1, 120.9 μM HBD2, 34 μM HBD3, and 13.8 μM LL37 when in contact with the keratinocytes. The amount of HBD3 deposited on the *S. aureus* in contact with the cells was well above the $BC_{99.9}$. In contrast, HBD2 and LL37 were only 25 and 32% of their respective $BC_{99.9}$. The level of HBD1 deposited on the bacteria would not be expected to affect viability under the conditions tested. Therefore, we concluded that HBD3 levels were sufficient to account for most of the killing by cultured keratinocytes, consistent with the observation of destruction and loss of viability in Figures 2 and 3. The values of equivalent *in vitro* exposure of the AMP are summarized in Table 3.

Exposure of *S. aureus* to human skin explants in a very similar assay was then performed to determine if the same AMPs were deposited on bacteria coming in contact with keratinocytes *in situ*. Four biopsies were collected from each of four normal donors and placed into KGM overnight at 37°C to allow for involution of damaged cells at the cut

Table 3. Exposure of *S. aureus* to AMP when in contact with keratinocytes in culture, and in human skin

Peptide	Exposed to cultured keratinocytes (±SEM, *n*=50 bacteria)	Exposed to human skin (±SEM, *n*=4 individuals)
LL37	13.8±3.0 μM	<5 μM[1]
HBD1	34.2±3.8 μM	37.2±8.3 μM[NS]
HBD2	120.9±30.1 μM	<5 μM[1]
HBD3	34±11.2 μM	86.9±9.3 μM[1]

AMP, antimicrobial peptide; NS, nonsignificant; *S. aureus*, *Staphylococcus aureus*.
[1]$P<0.05$ relative to keratinocytes in culture (Student's *t*-test).

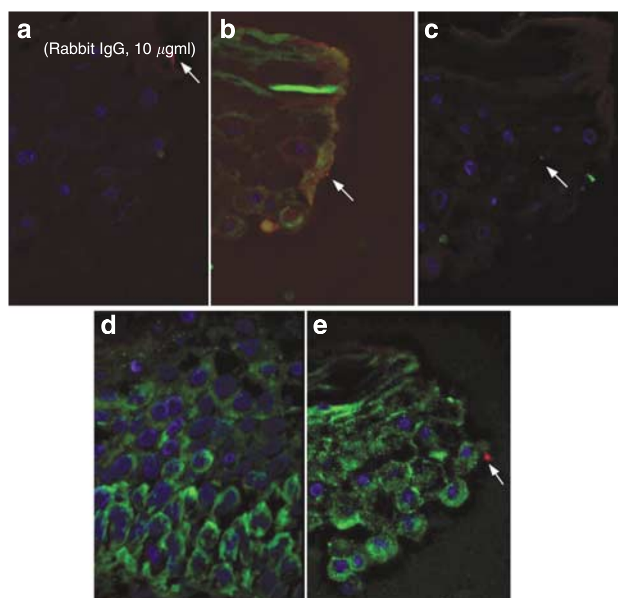

Figure 7. Expression of AMP and association of *S. aureus* (Wood 46) in human skin. (**a**) Skin from a representative donor out of four examined has been exposed to Texas Red-labeled *S. aureus* (Wood 46) (red, arrows), and immunostained with control antibody, or (**b**) antibodies specific for HBD1, (**c**) HBD2, (**d**) HBD3, or (**e**) LL37 (Green). Single confocal images show nuclei were counterstained with DAPI (blue). Bar = 20μm.

margins. *S. aureus* was then added to the tissues for 1 hour, after which the tissues were rinsed vigorously and prepared for imaging. Four biopsies were examined from each donor, with serial sections stained with control antibody, or antibody to each AMP of interest so that at least 10 bacteria in contact with the keratinocyte layers were quantitated for each sample/stain combination. In addition, specificity of the anti-HBD3 antibody was demonstrated by preabsorbing the antibody with synthetic HBD3 (Figure S3). Figure 7 shows that *S. aureus* were bound by keratinocytes in human skin in a manner similar to that of cultured keratinocytes, and that they expressed LL37, HBD1, and HBD3. HBD2 was only detected in rare cells. LL37, HBD1, and HBD3 rapidly became associated with *S. aureus* in contact with the keratinocytes, as shown in Figure 8. HBD1 was deposited on the bacteria equivalent to exposure *in vitro* at 37.2±8.34 μM, which was far below its $BC_{99.9}$ *in vitro*. HBD3 was deposited equivalent to *in vitro* exposure at 86.94±9.38 μM, which was approximately 8-fold higher than the amount required for efficient killing. LL37 was deposited poorly on the surface-bound bacteria, equivalent to less than 5 μM of *in vitro* exposure, which was the lower limit of quantitation. The kinetics and degree of HBD1 deposition were generally similar to that observed for the cultured keratinocytes, whereas HBD3 deposition was greater, and LL37 and HBD2 deposition were reduced. Therefore, only HBD3 was deposited in sufficient quantity for *S. aureus* killing by keratinocytes in skin. Exposure levels of *S. aureus* in contact with cultured keratinocytes, and keratinocytes in human skin are summarized in Table 3. However, in order to demonstrate that these AMPs were involved in the microbicidal mechanism, it was important to study the effect of their inhibition on the ability of keratinocytes to kill microbes.

To provide further data supporting constitutive expression of HBD3 in unstimulated human skin, we preformed quantitative real-time PCR to determine the number of copies of HBD3 mRNA per mg of human skin (punch biopsies). RNA extracted from skin biopsies of 12 individuals represented approximately 100,382±31,631 (SEM) copies of HBD3 RNA per mg of tissue. When compared to levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), there were an average of 41,831 copies of GAPDH mRNA for each copy of HBD3 mRNA in unstimulated skin. However, in some samples there were as many as one copy of HBD3 mRNA for 246 copies of GAPDH mRNA, and in others there were as few as one per 320,000. The median value was one copy of HBD3 mRNA per 2,080 copies of GAPDH, indicating significant expression of the HBD3 gene in the absence of induction. These data are summarized in Table 4.

In order to determine which peptides were necessary for killing of *S. aureus*, we conducted the killing experiments in the presence of antibodies to each of the AMP of interest, to determine the effect of preventing them from binding to the bacteria in contact with the cells. Specificity of each antibody for the target AMP was determined in preliminary experiments by using the antibodies to block killing of *S. aureus* exposed to the various purified AMP in KGM *in vitro*. The antibodies each blocked killing by their cognate peptide, but not to any of the other peptides. The effects of the antibodies on the ability of keratinocytes to kill *S. aureus* are shown in Figure 9. These results show that antibodies to human neutrophil peptide 1, which is not present in keratinocytes and serves as a control, did not significantly inhibit killing of the bacteria. Similarly, antagonism of LL37 and HBD1 had no significant effect on the killing ability of the keratinocytes. However, when antibodies to HBD2 or HBD3 were added to the cultures, concentration-dependent inhibition was observed (Figure 9). The highest concentration of antibodies to HBD2 and HBD3 reduced *Staphylococcus* killing by 25 and 75%, respectively, confirming that these two peptides are not only deposited at sufficient concentration on cell-associated bacteria to account for killing, but they are also necessary for

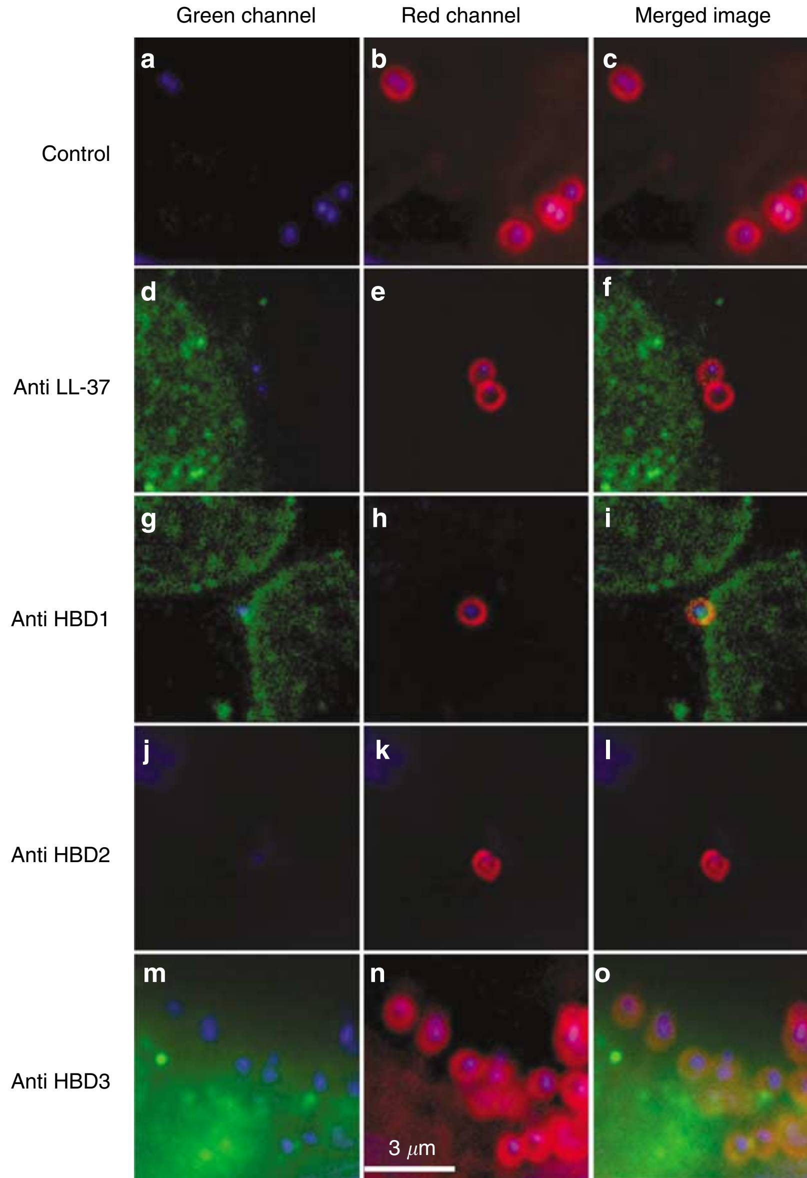

Figure 8. Accumulation of AMP on *S. aureus* in association with keratinocytes *in situ*. Skin from a representative donor, out of four examined has been exposed to Texas Red-labeled *S. aureus* (red, arrows), and immunostained with (**a–c**) isotype control antibody, or antibodies specific for (**d–f**) LL37, (**g–i**) HBD1, (**j–l**) HBD2, or (**m–o**) HBD3 (Green). In the single confocal planes, green fluorescence overlapping the red bacteria indicates deposition of the AMP on the bacteria. Constrained iterative deconvolution was used for these images in order to highlight position of the fluorescence. Therefore, intensities are not to scale. Nuclei were counterstained with DAPI (blue).

most of the killing of cell-associated *S. aureus*. In addition, as killing could be inhibited by extracellular antibody, killing likely takes place at the cell surface, confirming that internalization of the bacteria is not required for killing to occur (Figure 4).

DISCUSSION

Although it is known that induced (time course of >24 hours) AMPs play an important role in innate immune response to microbes, the immediate response of skin keratinocytes during initial invasion of microbes has not been well studied. In these studies, we have shown that human keratinocytes possess potent constitutive antimicrobial activity, which kills and eliminates *S. aureus* from the skin within 1 hour of contact. The speed and potency with which cultured keratinocytes were able to kill bacteria suggest that these cells possess a previously unrecognized microbicidal capacity, which may contribute substantially to protection of the epidermis, and hence the entire organism, from invasion by microbes.

Previous studies have not considered this constitutive activity, focusing instead on gene expression induced by contact with microbes or microbial products (Braff *et al.*, 2005; Menzies and Kenoyer, 2005, 2006; Wehkamp *et al.*,

Table 4. Expression of mRNA encoding GAPDH and HBD3 mRNA in skin

mRNA encoding	Average copies per mg (±SEM, *n*=9)	Range
GAPDH	$4.6\times10^{9}\pm3.5\times10^{8}$/mg	1.5×10^{6}–3.9×10^{10}
HBD3	$1\times10^{5}\pm3.2\times10^{4}$/mg	2.4×10^{3}–3.4×10^{5}

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBD, human beta-defensin.

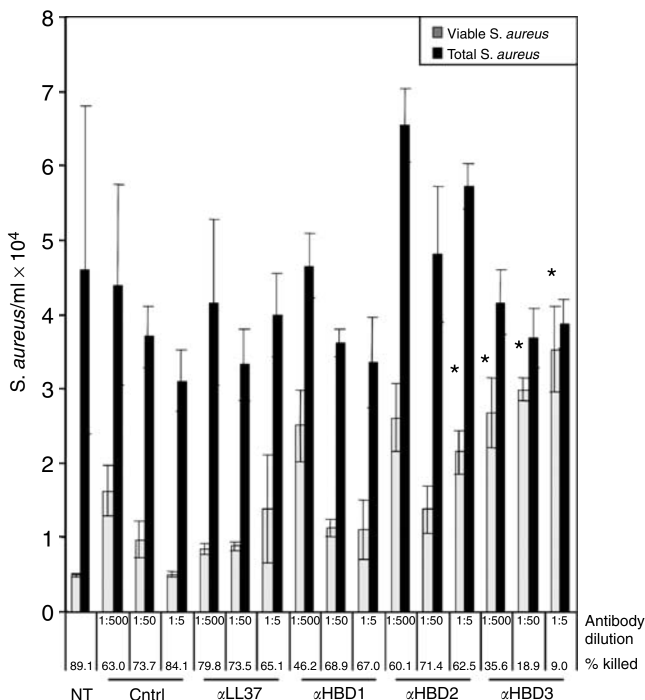

Figure 9. Inhibition of keratinocyte killing of *S. aureus* by antibodies to AMP. *S. aureus* were exposed to keratinocytes for 1 hour in the presence of increasing concentrations of control antibody, or antibodies to LL37, HBD1, HBD2, or HBD3. After washing, the monolayers were lysed and total bacteria determined radiometrically, whereas viable bacteria were determined by colony-forming unit. The number of total cell-associated and viable cell-associated bacteria (*y* axis) was plotted against the dilution of each antibody (*x* axis). The percentage of each bacteria culture killed was calculated from the total *versus* total viable data, and is presented below the *x* axis. $^{*}P<0.01$ relative to control antibody. Data are representative of three experiments.

2006). However, the constitutive activity reported herein could make important contributions to limiting bacterial growth following initial penetration of the stratum corneum, such as after scratching or abrasions that would expose the middle of the epidermis to the outside world, thereby limiting inflammation and infection. Keratinocytes are known to produce potently microbicidal molecules, including three β-defensins (Ali *et al.*, 2001), cathelicidin fragments (Sharlow *et al.*, 2000), and RNIs. However, no mechanism has previously been shown for how these AMPs were utilized by the cells to kill microbes (Kroll *et al.*, 1999) such as *S. aureus*. Our current experiments illustrate how these defensins contribute to a previously unappreciated active host defense role for keratinocytes.

As factors secreted into the medium had no effect on the viability of microbes, binding of the microbes to the cells played an important role in the killing mechanism. *S. aureus* is known to bind keratinocytes efficiently in the absence of opsonization, via fibronectin binding proteins interacting with integrin α5β1 on the keratinocytes (Murakami *et al.*, 2004). We noted that exposure of cultured keratinocytes to *S. aureus* resulted in rapid binding of bacteria to the cells, followed by internalization by keratinocytes, which has been previously noted as a virulence mechanism for *S. aureus*, as the bacteria escape from the endosomes and cause apoptosis of the keratinocytes (Bayles *et al.*, 1998; Kintarak *et al.*, 2004). The kinetics of internalization also showed apparent disappearance of the covalently attached Texas Red fluorophore from viable bacteria. This could have been due to destruction of the bacteria, and subsequent release of the labeled macromolecules from the cells. An alternative interpretation would be that the bacteria remained intact, but that the label became non-uniformly distributed on the bacteria because of cell division, or that the label and the associated macromolecules were shed from the bacteria. However, as our results indicate that *S. aureus* has a high level of spontaneous binding to keratinocytes, and that this contact results in loss of the majority of bacterial viability (Figure 3), we believe that the diffuse fluorescence in the cells after 1 hour represents bacteria which have been destroyed.

In addition to binding via the α5β1 receptor (West *et al.*, 1989), keratinocytes have receptors normally associated with professional phagocytes, FcγRI, FcγRII, and FcγRIII (Tigalonowa *et al.*, 1990; Bjerke *et al.*, 1994), which can be upregulated by cytokine stimulation (Mempel *et al.*, 2002). This suggests that binding and killing of microbes by keratinocytes shares important similarities with professional phagocytes. Inhibition of *S. aureus* internalization by keratinocytes by both amiloride and colchicine indicates that both macropinocytosis and phagocytosis are important internalization pathways for keratinocytes, and is not simply a passive invasion process as previously suggested (Aufiero *et al.*, 2004). The conclusions we draw from these studies differ from the conclusions of others for two reasons: first, we have cultured the keratinocytes in higher levels of Ca^{2+}, which results in differentiation of the cells to a more mature phenotype (Stanley and Yuspa, 1983). Second, we are calculating the loss of bacterial viability from the initial point of inoculation, rather than from the point of internalization by the cells. It is clear from our results that the majority of bacterial killing occurs before internalization when the keratinocytes are encouraged to differentiate. Therefore, we conclude that like macrophages and neutrophils, human keratinocytes are microbicidal phagocytes. However, even though killing and internalization both occurred, the experiments shown in Figure 4 clearly demonstrate that internalization is not required for the microbicidal mechanism. In addition, the killing mechanism could be inhibited via extracellular antibodies to HBD2 and HBD3 (Figure 9). Despite this, internalization may still be important in limiting

inflammation, as *S. aureus* contains several molecules known to be potently inflammatory (Tokura *et al*., 1994; Ezepchuk *et al*., 1996; Mempel *et al*., 2003; Matsubara *et al*., 2004). Therefore, internalization of *S. aureus* by keratinocytes may limit inflammation by sequestration of inflammatory substances. In addition, the constitutive microbicidal capacity of keratinocytes is not likely to be limited to *S. aureus*. Glaser *et al*. (2005) demonstrated that psoriasin is mainly responsible for preventing *E. coli* from colonizing the skin. It is possible that internalization is important for killing *S. aureus* that survive the initial encounter with the cell surface, or for killing other microbes. Indeed, Braff *et al*. (2005) demonstrated that the viability of *S. aureus* declined for several hours after internalization.

HBD2 and HBD3 expression has not been widely reported in healthy skin (Nomura *et al*., 2003; Sorensen *et al*., 2006). Indeed, we were not able to detect HBD2 in skin biopsies, confirming the observations of Harder *et al*. (1997) that HBD2 is expressed at very low levels in normal skin, although HBD2 has been shown to be quite important for protecting the epithelium against many different bacteria (Chung and Dale, 2004). However, using confocal microscopy and quantitative real-time PCR we have shown that HBD3 in particular is expressed at low levels in normal skin (Nomura *et al*., 2003), confirming the observation of Sorensen *et al*. (2006), in which HBD3 was clearly detected in normal skin by Western analysis. Although the constitutive expression of HBD3 maintains an average of 1×10^5 mRNA copies per mg of skin, staining of the skin for HBD3 peptide reveals that accumulation is not uniform. Therefore, HBD3 mRNA levels may be substantially higher than the average in those cells of the skin samples, which express the peptide.

Although Sorensen *et al*. (2006) have shown that simple wounding of the skin can stimulate HBD3 expression via transactivation of the EGF receptor, this process requires 3–4 days to significantly elevate mRNA for HBD3. If the innate immune response which contains *S. aureus* infections truly required 3–4 days for induction (Sorensen *et al*., 2006), *S. aureus* would be expected to cause far more morbidity and mortality than it currently does, as a large percentage of people are colonized with *S. aureus* (Fridkin *et al*., 2005). Therefore, the hypothesis that there exists an innate mechanism to sterilize small inoculae of bacteria into the epidermis is reasonable.

Our investigation of the microbicidal mechanism of keratinocytes suggested a model in which AMP stored in the cells was mobilized onto the bacteria (Figure 6), which occurred at the same time as internalization. Mobilization of vesicles containing antimicrobial substances into phagosomes containing microbes is a hallmark of bacterial killing by professional phagocytes. Fusion of granules with phagosomes in neutrophils allows application of antimicrobial substances contained in them with minimal dilution, as compared with extracellular secretion (Cauza *et al*., 2002). Binding and internalization of *S. aureus* by keratinocytes associated with fusion of vesicles containing defensins may account for the requirement for contact between the bacteria and the cells, as the same amount of defensin secreted into the medium was too dilute to be effective. Indeed, *S. aureus* exposed to cell-free medium harvested from keratinocyte cultures exposed to *S. aureus* were unaffected by the conditioned medium. Therefore, binding and internalization of *S. aureus* by the keratinocytes may allow exposure to concentrated AMP, which could enhance their antimicrobial efficacy.

In order to link our observations with isolated keratinocytes to processes, which operate in human skin, we examined the ability of keratinocytes in skin to bind, internalize, and mobilize defensins onto bacteria. The punch skin biopsies used created a surface containing cells, which were bisected by the instrument. However, after 24 hours in culture, the dead cells had been replaced by viable keratinocytes at the surface. The surface keratinocytes were indeed able to bind and internalize *S. aureus*, consistent with our observations in tissue culture. In addition, AMPs were mobilized onto the bacteria. However, although HBD1, HBD3, and LL37 were present in most of the keratinocytes, only HBD3 was mobilized at microbicidal levels onto *S. aureus*, which came into contact with the cells. Therefore, it is likely that the antimicrobial mechanism operative in cultured keratinocytes also works in human skin. However, it was not possible to make a causal link between accumulation of defensins on the bound *S. aureus* and the microbicidal mechanism without conducting depletion studies, to determine if inhibition of defensin activity resulted in inhibition of bacterial killing.

Glaser *et al*. (2005) were able to demonstrate that psoriasin was critical for preventing colonization of skin by *E. coli* by antagonizing that peptide with antibodies, and observing the effect on bacterial viability. We employed a similar technique, depleting LL37, HBD1, HBD2, and HBD3 from keratinocyte cultures using specific antisera. This revealed that killing could be substantially inhibited by antagonizing HBD2 and HBD3, but not HBD1 or LL37. This confirms that the rapid *S. aureus* killing mechanism of cultured keratinocyte operates in a compartment accessible to antibodies, and involves HBD2 and HBD3. In human skin explants, bacteria which came into contact with the keratinocytes accumulated approximately twice as much HBD3 as those in contact with cultured keratinocytes. However, during the 1 hour time frame of rapid killing, LL37 was not deposited on the bacteria in significant amounts by skin cells. Also, in contrast to cultured keratinocytes, HBD2 was expressed by very few keratinocytes in the skin explants, and *S. aureus* were not observed in contact with those cells. This difference between AMP expression by cultured keratinocytes in comparison with keratinocytes in skin is likely due to differentiation of the cells in culture using defined medium, which does not perfectly reflect the *in vivo* situation in skin. However, as the contribution of HBD2 to the killing mechanism in culture was considerably less than that of HBD3, and the levels of HBD3 in the skin cells were approximately twice that in cultured keratinocytes, the increased HBD3 likely compensates for the lack of HBD2. Therefore, we conclude that HBD3 is both necessary and sufficient to account for the constitutive ability of human keratinocytes to kill *S. aureus* within minutes of contact with the cell surface. As we were unable to measure killing of

S. aureus in contact with skin *in situ*, it is possible that other host defense molecules play a role in early killing by keratinocytes in skin, such as lysozyme or RNase 7 (Harder and Schroder, 2002). However, the average level of HBD3 deposited on *S. aureus* by keratinocytes in skin suggests that killing should be quite rapid even in the absence of other mediators. It is important to note that not all of the bacteria in contact with the skin were exposed to sufficient HBD3 to suggest killing. Those bacteria at the lower end of the exposure distribution might have had high levels of other host defense molecules, which we have not examined in these experiments. It is likely that complete sterilization of an inoculation of *S. aureus* into the keratinocyte layers or beyond would require multiple, complementary mechanisms, deposition of HBD3 being only one of them.

In summary, our data demonstrate that human keratinocytes possess a potent and previously unrecognized constitutive mechanism to rapidly kill *S. aureus*, which come into contact with them. The mechanism involves association of the bacteria with the cell surface, followed by elaboration of AMP onto the bacteria. The most important of the AMP for killing of *S. aureus* by the cultured keratinocytes were HBD2 and HBD3, as evidenced by the ability of antibodies to these two AMP to inhibit killing when added to the medium. Examination of this process in samples of human skin revealed that HBD3 mRNA and peptide was expressed in healthy skin, and deposited on cell-associated *S. aureus* at twice the level observed in cultured keratinocytes, whereas HBD2 did not appear to participate in killing by keratinocytes in skin. Despite operation of the killing mechanism at the cell surface, the remains of the dead and dying bacteria are rapidly internalized by the keratinocytes. This may serve to limit inflammation by sequestering proinflammatory substances born by *S. aureus*. Therefore, keratinocytes contribute to innate immune defense of the host by directly killing bacteria utilizing HBD3, and sequestration of the products via internalization.

MATERIALS AND METHODS

Reagents

LL37 was synthesized by the Molecular Resource Center at National Jewish Medical and Research Center (NJMRC; Denver, CO) using standard T-boc reagents, and purified to homogeneity via reversed phase-HPLC. HBD1, HBD2, and HBD3 were the generous gifts of Wuyuan Lu, University of Maryland. Human neutrophil peptide 1 was the gift of Jacek Lubkowski, National Cancer Institute. All peptides were resuspended at 1 mg/ml, in 0.01% acetic acid, aliquoted and stored at -80°C until use. l-NAME, 4′,6-diamidino-2-phenylindole (DAPI), and *o*-phenylene diamine were obtained from Sigma (St Louis, MO).

Skin biopsies

The skin of normal healthy donors, not taking systemic or topical medications, was harvested following provision of informed consent as per a protocol approved and monitored by the NJMRC institutional review board, in accordance with the Declaration of Helsinki Principles. Four 2 mm skin biopsies were collected from each donor, and cultured overnight at 37°C, 5% CO_2 to allow for involution of damaged cells at the cut edge of the tissue. The biopsies were then exposed to *S. aureus* (Wood 46) labeled with Texas Red for 5–60 minutes, after which they were rinsed three times with KGM, and fixed with 4% paraformaldehyde.

Keratinocytes

Normal human neonatal keratinocytes were obtained from Cascade Biologics (Portland, OR). The cells were cultured in EpiLife basal medium supplemented with HKGS-V2 (Cascade Biologics). The cells were frozen at passage two, and used for these studies at passage three. The cells were cultured at 5×10^3 in coated (Coating Matrix, Cascade Biologics) 24-well tissue culture plates. When the cells reached 50% confluence, the medium was changed and $CaCl_2$ was added to 1.3 mM in order to induce differentiation (Stanley and Yuspa, 1983). The cells were then cultured an additional 5 days, changing the medium every other day before use. The human keratinocyte-like cell line HaCaT was obtained from American Type Culture Collection (ATCC, Rockville, MD), and maintained in DMEM containing 10% fetal bovine serum, L-glutamine, penicillin/streptomycin, $1\times$ minimal essential medium vitamin solution (Gibco-Invitrogen, Carlsbad, CA), and non-essential amino acids (Gibco-Invitrogen). Twenty-four hours before a killing assay, the cells were seeded into 24-well plates at approximately 2×10^5 cells/ml, and allowed to incubate overnight with antibiotic-free minimal essential medium at 37°C. At the time of the killing assays, the wells contained $4\text{–}5 \times 10^5$ cells each.

Neutrophils

Human neutrophils were isolated from normal healthy individuals according to a protocol reviewed and approved by the Institutional Review Board (IRB) using a Percoll (Pharmacia, Piscataway, NJ) density gradient (Sala *et al.*, 1999). In brief, 4.4 ml of 3.8% (w/v) sodium citrate (Fisher Scientific, Pittsburgh, PA) was added to 40 ml of heparinized blood. The blood was then centrifuged at $400 \times g$ for 20 min. The plasma layer was then removed, 5 ml of 6% (w/v) dextran (Pharmacia) was then added to the pelleted whole blood, the total volume was brought up to 50 ml with saline and mixed gently. The cell suspension was then left for 30 minutes at room temperature to allow the red blood cells to settle. The upper white blood cell layer was removed, centrifuged at $400 \times g$ for 10 minutes, the supernatant discarded and the pellet resuspended in 2 ml of autologous plasma. The cell suspension was then under-laid with a 42% (w/v) followed by a 51% (w/v) Percoll gradient and centrifuged at $350 \times g$ for 10 minutes. The resulting neutrophil-rich layer was carefully removed. Neutrophils were then resuspended in phosphate-buffered saline (PBS), centrifuged at $350 \times g$ for 10 minutes, and the supernatant discarded. The resulting neutrophil pellet was then resuspended in HBSS.

S. aureus

S. aureus ATCC strain numbers 29213 and 10832 (Wood 46) were employed in our experiments. *S. aureus* was grown overnight in tryptic soy broth at 37°C with aeration. *S. aureus* was radioactively labeled with 0.01 mCi ^{3}H uracil (Amersham Pharmacia, Piscataway, NJ). For each experiment, the bacterial cells were washed three times by centrifugation, and resuspension in $1\times$ PBS. Opsonization of *S. aureus* was carried out at 37°C with either antibody opsonization at a 1:1,000 dilution with mouse Anti-Staph A

(Research Diagnostics Inc., Flander, NJ) and/or serum opsonized with 10% heat-inactivated human plasma obtained from healthy donors. Bacterial concentration was determined by optical density reading of the bacteria measured in a Beckman spectrophotometer.

Bactericidal assay

Twenty-four hours before the experiment, the cell culture medium was replaced with antibiotic-free media containing all supplements and 1.3 mM $CaCl_2$. At the time of the experiment, fresh antibiotic-free media with 1×10^6 bacteria/ml was inoculated into each well. Cells were co-incubated with bacteria for 1 hour at 37°C under 5% CO_2, rinsed three times with 1 × PBS and the monolayers were detached from the plates by adding 500 μl of trypsin/EDTA solution 1 × (Cascade Biologics). Following detachment from the plate 500 μl of trypsin neutralizing solution 1 × (Cascade Biologics) was added. The cells were lysed by resuspension in 500 μl of sterile deionized water. The cell lysates were quantified for total *S. aureus* by radioactive decay of ^{3}H measured by a Beckman scintillation counter, and serially diluted 10- to 1,000-fold and plated onto tryptic soy agar plates to quantify the viable, cell associated *S. aureus* upon formation of colonies.

For studies examining inhibition of bactericidal activity by keratinocytes, inhibitors such as l-NAME, colchicine, amiloride, or antibodies to AMPs were added to the cell culture medium at various concentrations 1 hour before addition of bacteria. The bacteria were then added, incubated, and the percentage of viable bacteria determined after 1 hour as above.

Internalization assay

The assays were performed as described above with the following alterations: the bacteria were not labeled with ^{3}H uracil. After the 1 hour incubation at 37°C under 5% CO_2, KGM supplemented with 100 μg/ml gentamicin was added to each well and incubated for an additional hour to kill any extracellular *S. aureus*. The cultures were then rinsed three times, lysed, and bacteria plated to determine the number of viable colonies as described above. The data represent total viable bacteria, which survive gentamicin, implying that they were protected within the cells.

Imaging

The Woods strain, ATCC 10832 was used for imaging, due to markedly reduced nonspecific antibody binding (Gross *et al.*, 1978). The bacteria were surface labeled with 10 μM Texas Red, succinimidyl ester in PBS for 30 minutes at 37°C, which results in a covalent bond between bacterial surface macromolecules and the fluorophore. The unbound label was then removed by centrifugation and resuspension in 1 × tryptic soy broth three times. The density of the bacteria was then estimated by absorbance as above. Keratinocytes were cultivated and differentiated as above in 24-well plates containing 12 mm round no. 1 coverglasses (Fisher Scientific). The fluorescently labeled bacteria were then added to the keratinocyte cultures for intervals of 1 minute to 1 hour, after which the unbound bacteria were removed by vigorous rinsing, and the cultures fixed with 4% paraformaldehyde overnight at 4°C.

For staining with antibodies to AMPs, samples were permeabilized and blocked with PBS containing 3% BSA, 5% human serum, and 0.1% Tween 20 (staining buffer). Polyclonal rabbit anti-HBD1 at 10 μg/ml (Alpha Diagnostics, San Antonio, TX, 1:100), polyclonal rabbit anti-HBD2 at 2.5 μg/ml (Alpha Diagnostics, 1:250), polyclonal rabbit anti-HBD3 at 10 μg/ml HBD3 (Orbigen, San Diego, CA, 1:100), and polyclonal rabbit anti-LL37 at 10 μg/ml (HyCult Biotechnology, 1:100), or preimmune serum were diluted as noted into staining buffer, and incubated with the samples overnight at 4°C. After washing to remove unbound antibody, the samples were developed with polyclonal goat anti-rabbit antibody conjugated to FITC. The samples were then counterstained with wheat germ agglutinin-Cy-5 (Molecular Probes, Eugene, OR) and DAPI. After staining, the coverslips were mounted on glass slides with the attached cells toward the slide using 3% *o*-phenylene diamine in 90% glycerol, 1 × PBS as an antifade and mounting medium. The samples were then imaged using a digital imaging workstation (Zeiss Axiovert 200 M microscope; Carl Zeiss Microimaging Inc., Thomwood, NY) controlled by Slidebook software (Intelligent Imaging Innovations, Denver, CO). A series of images in the *z* axis was collected at intervals of 0.2 μm, until the feature of interest was completely captured. Images were deblurred using nearest neighbors deconvolution (Kisich *et al.*, 2002).

Quantitation of mRNA levels in normal skin

Total RNA was isolated from normal skin explants by chloroform:-phenol extraction and isopropanol precipitation according to manufacturer's guidelines (Sigma Chemical Co., St Louis, MO). RNeasy Mini Kits (Qiagen, Valencia, CA) were used according to the manufacturer's protocol to isolate RNA from cell cultures and to further purify RNA from skin explants. One microgram of RNA was reverse transcribed in a 20 μl reaction containing Random Primers (Invitrogen, Carlsbad, CA), RNase Inhibitor (Invitrogen), and Superscript III enzyme (Invitrogen). Real-time PCR was performed and analyzed by the dual-labeled fluorigenic probe method using an ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, CA). Primers and probes for human GAPDH were purchased from Applied Biosystems. HBD3 primer and probes were prepared as described previously (Nomura *et al.*, 2003). The TaqMan probes were purchased from Applied Biosystems and were 5′ labeled with 6-carboxyfluorescein (FAM) and 3′-labeled with 6-carboxy-tetramethylrhodamine (TAMRA). Amplification reactions were performed in MicroAmp optical tubes (Applied Biosystems) in a 25 μl volume containing 2 × TaqMan Master Mix (Applied Biosystems), 900 nM forward primer, 900 nM reverse primer, 200 nM probe, and the template RNA. Thermal cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes for one cycle. Subsequently, 40 cycles of amplification were performed at 94°C for 15 seconds and 60°C for 1 minute. Standard curves were generated from GAPDH and HBD3 mRNA synthesized via *in vitro* transcription, according to the manufacturer's instructions (T7 megascript, Ambion, Austin, TX).

Quantitation of AMP exposure in cultured keratinocytes

Intensity values of at least 50 bacteria per concentration were used to construct standard intensity *versus* exposure curves for LL37, HBD1, HBD2, and HBD3. The intensity values after subtraction of the average background of samples exposed to control antibody were fit to lines using regression analysis. The line was considered a valid representation of the data if a correlation coefficient (R^2) of 0.95 or greater was reported. Keratinocyte samples stained and imaged in the same experiment as the standard samples were imaged using identical image capture settings as the standards. At least 20 bacteria

in each of triplicate wells were imaged for each AMP stain, each bacterium's intensity was interpolated to equivalent *in vitro* AMP exposure via the respective line equations. Equivalent exposure values were then compared with samples stained with the appropriate control antibody via *t*-test.

Quantitation of AMP exposure in human skin was analyzed using similar techniques as above, with the exception that four biopsies were examined from each of four individuals. Serial sections from each biopsy were stained and imaged until at least 10 bacteria in contact with the keratinocyte layers were observed for each biopsy, for a total of 40 bacteria per AMP stain per individual. Intensities of each bacterium were interpolated to equivalent AMP exposure via the standard curves, and the values were compared to bacteria in serial sections stained with control antibody. Average equivalent *in vitro* exposure values for each stain were compared with control antibody via *t*-test, and a $P<0.05$ was considered significant.

Statistical analyses

S. aureus killing assays were analyzed by comparing the means of total *versus* viable bacteria from triplicate samples receiving different treatments using *t*-test. A $P<0.05$ was considered significant. Each comparison was performed three times.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. Cross sections of a three-dimensional keratinocyte data set.

Figure S2. Effect of duration of antibiotic washout on keratinocyte killing of *S. aureus*.

Figure S3. Specificity of anti-HBD3 antibody staining.

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