EFFECTS OF STAPHYLOCOCCUS AUREUS ON HUMAN KERATINOCYTES

by

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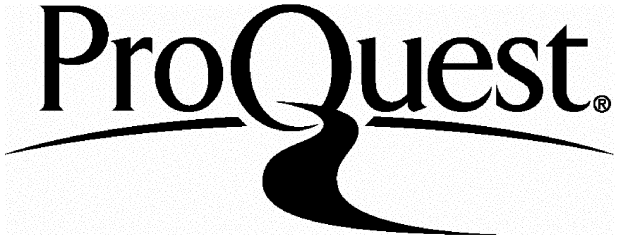

Abstract

In healing wounds, keratinocytes migrate on a fibronectin (FN)-rich granulation tissue and may phagocytose FN-coated wound debris. *Staphylococcus aureus* is a common infecting bacterium in wounds and heavy colonization of toxin-producing strain may cause mucosal damage. In addition, *S. aureus* can be internalized by non-phagocytic host cells thought to facilitate evasion of host cell immunity and antibiotics.

The purpose of the experiments described in this thesis was to investigate the bacterial and host factors influencing the adhesion and internalization of *S. aureus* by human keratinocytes as well as the effects of *S. aureus* fibronectin-binding proteins (FnBPs) and its secreted products on keratinocyte functions.

S. aureus FnBPs and host cell integrin α5β1 were important for the internalization of *S. aureus* by immortalized keratinocyte cell lines. However, in primary keratinocytes, *S. aureus* internalization occurred independently of FnBPs. In addition, purified *S. aureus* FnBP (rFnBPBD1-D4) had no effects on the adhesion and migration of primary keratinocytes on the fibronectin substrate, however it inhibited the migration of immortalized UP keratinocytes. Decreased UP cell migration in the presence of rFnBPBD1-D4 was not due to decrease in the adhesion or proliferation (BrdU incorporation) of the cells. The difference in the response of primary cells and cell lines to *S. aureus* may have important implications for the interpretation and translation of *in vitro* studies to predict *in vivo* responses.

The effects of culture supernatants from *S. aureus* on keratinocyte functions were strain- and growth phase-dependent. The culture supernatants that contained undetectable toxins such as α- and β-toxin did not have any deleterious effects on keratinocytes. Toxin-containing supernatants, particularly α-toxin, from stationary growth phase of *S. aureus* inhibited the adhesion and migration of keratinocytes and increased cell death. *S. aureus* may gain access to the submucosal tissues by fatal damage of keratinocytes with its cytotoxins.

The rFnBPBD1-D4 inhibited endogenous FN polymerization. Interaction of rFnBPBD1-D4 with intact and fragments of FN may influence cell behaviour.

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Abbreviations

α_2M	α_2-macroglobulin
ATP	adenosine triphosphate
5-BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
CS	culture supernatant (from *S. aureus*)
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulphoxide
E-64	L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane
ECM	extracellular matrix
EDA	extradomain A of fibronectin
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Em	erythromycin
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FG	fibrinogen
FN	fibronectin
FnBPs	fibronectin-binding proteins
Ham's F-12	Nutrient Mixture Ham's F-12
HUVEC	human umbilical vein endothelial cell
ICAM	intercellular adhesion molecule
IL	interleukin
IPTG	isopropylthio-β-D-galactoside
KGM	keratinocyte growth medium
LB	Luria-Bertani medium
LM	laminin
mAb	monoclonal antibody
MHC	major histocompatibility complex
MSCRAMMs	microbial surface components recognizing adhesive matrix molecules
NHK	normal human primary oral keratinocyte

PBS	phosphate buffered saline
PECAM	platelet endothelial cell adhesion molecule
PMSF	phenylmethanesulphonyl fluoride
rFnBPBD1-D4	recombinant protein encompassing D1-D4 repeat units of *S. aureus* FnBPB
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel eletrophoresis
SFM	serum-free medium (DMEM:Ham's F-12, 3:1)
Tc	tetracycline
TGF	transforming growth factor
THB	Todd Hewitt broth
TNF	tumour necrosis factor
TSST-1	toxic shock syndrome toxin-1
VCAM	vascular cell adhesion molecule

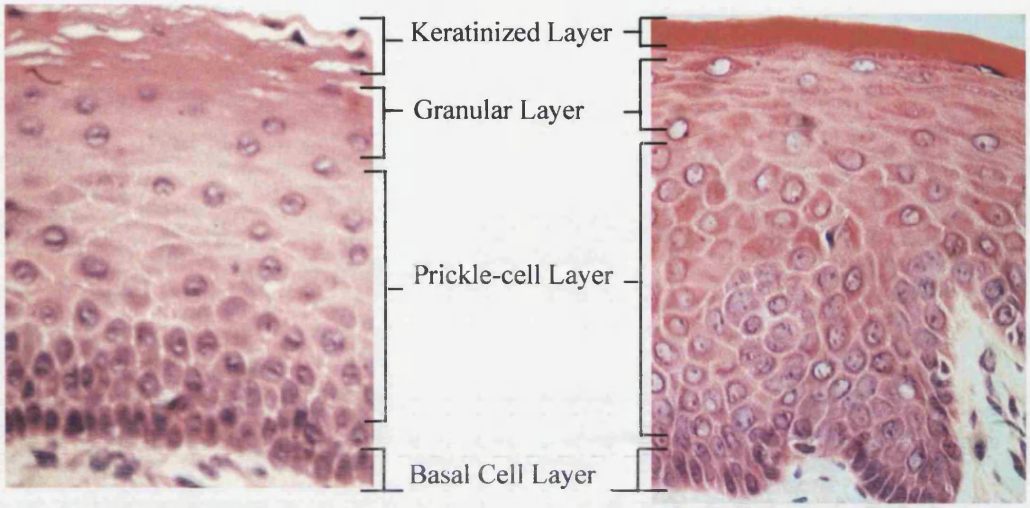
Chapter 1
Literature Review

1.1 Oral Epithelium

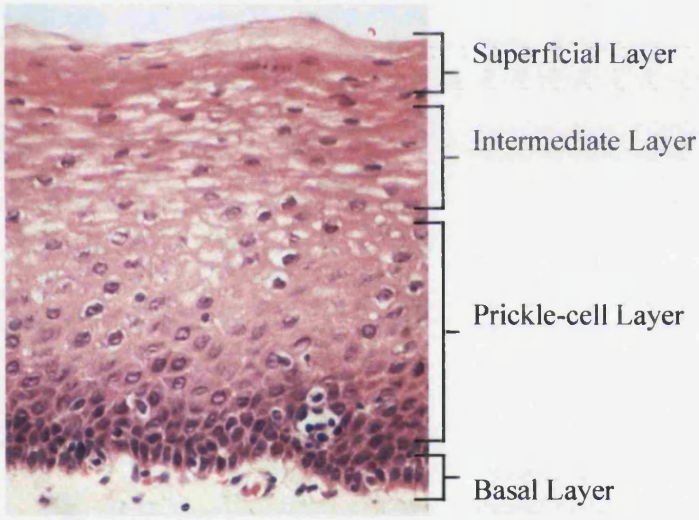
The oral mucosa, the skin and the intestinal lining consist of two separate tissue components: a covering epithelium and an underlying connective tissue. Whereas the skin is dry and provides the covering for the external surface of the body, the oral or the intestinal mucosa is lined with a moist mucosa (mucous membrane). The oral epithelium represents the primary barrier between the oral environment and the deeper tissues.

1.1.1 Principal Structure of Oral Epithelium

Like the epidermis, the oral epithelium is a stratified squamous epithelium mainly composed of a single cell type, the keratinocyte. The keratinocytes are tightly attached to each other and arranged in a number of distinct layers or strata (Figure 1-1). The basal layer (stratum basale) is a layer of cuboidal or columnar cells adjacent to the basement membrane. Above the basal layer are several rows of larger polygonal cells known as the prickle-cell layer (stratum spinosum). Histological examination reveals the cells in the stratum spinosum shrinking away from one another, remaining in contact only at points known as the intercellular bridges, or desmosomes. In keratinized oral epithelium, the next two layers are the granular cell layer and the keratinized layer. The granular cell layer (stratum granulosum) consists of large, flattened cells containing numbers of small basophilic granules called keratohyalin granules. The keratinized layer (stratum corneum) is the surface layer comprising of very flat, eosinophilic cells with or without pyknotic nuclei. In non-keratinized oral epithelium, there are no sudden changes in the appearance of cells above the prickle-cell layer. The outer half of the tissue is divided rather arbitrarily into two zones, the intermediate layer (stratum intermedium) and the superficial layer (stratum superficiale). The granular cell layer is not present in non-keratinized epithelium. The superficial layer does not stain with eosin as does the surface of keratinized epithelium, and contains cells with plump nuclei.



(a) Gingival mucosa (b) Palatal mucosa



(c) Buccal mucosa

Figure 1-1 Histological sections of the main types of epithelial maturation in human oral epithelium. Original magnification: x400 for (a) and (b); x200 for (c).

(a) Parakeratinized epithelium in gingiva. The keratin squames retain pyknotic nuclei and the granular cell layer contains only a few scattered granules.

(b) Orthokeratinized epithelium in the hard palate. The granular layer is more prominent.

(c) Nonkeratinized epithelium in buccal mucosa. There is no clear division of the epithelium into strata and nuclei are apparent in the surface layer.

1.1.2 Epithelial Proliferation and Maturation

The structural integrity of the epithelium is maintained by a self-renewal process in which cells produced by mitosis in the deeper layers migrate to the surface to replace those that are shed. The epithelial cells can thus be considered to consist of two functional populations: a progenitor population and a maturing population (Squier and Finkelstein, 1998). The maturing population is continually undergoing a process of differentiation or maturation to form a protective surface layer. The progenitor cells divide and provide new cells. They are located in the basal layer in thin epithelia (*e.g.* the floor of the mouth) and in the lower two to three cell layers in thicker epithelia (*e.g.* cheek and palate). The progenitor population is not homogeneous. It consists of two functionally distinct sub-populations: a small portion of stem cells and a larger portion of amplifying cells. The stem cells have a very slow cell cycle. They produce basal cells and retain the proliferation potential of the tissue. The amplifying cells proliferate to increase the number of cells available for subsequent maturation with terminal differentiation as they move to the surface. Factors affecting epithelial proliferation and differentiation include epidermal growth factor (EGF), keratinocyte growth factor (KGF), interleukin (IL)-1, and transforming growth factor (TGF)-α and TGF-β.

1.1.3 Epithelial Cohesion and Adhesion

Cohesion or connection between cells of the oral epithelium is provided by various intercellular materials including desmosomes, cadherins, gap and tight junctions. Desmosomes are relatively few in the basal layer but are increased in the suprabasal and spinous cell layer.

The basal keratinocytes rest on a basement membrane which appears as a structureless eosinophilic band in hematoxylin and eosin stained histologic sections. It is composed of a specific subset of extracellular matrix (ECM) proteins including type IV collagen, laminin and heparan sulfate proteoglycans. In addition, other recognized components of basement membrane include the hemidesmosomes, which contain bullous pemphigoid antigens; anchoring filaments which contain laminin-5 (also known as kalinin, epiligrin and nicein); anchoring fibrils which contain type

VII collagen; and entactin (Larjava *et al*, 1996; Bartold *et al*, 2000). The firm adhesion of the basal layer to the substratum is mediated by hemidesmosomes and focal adhesions present on the basal membranes of the basal keratinocytes. Integrins are the primary adhesion receptors present in both hemidesmosomes and focal adhesions. Integrin α6β4 is a component of hemidesmosomes and binds to laminins 1 and 5 (De Luca *et al*, 1990; Niesson *et al*, 1994).

1.2 Integrins

Integrins are a family of cell surface proteins that mediate cell-cell and cell-ECM adhesion. Adhesion is of fundamental importance to a cell; it provides anchorage, cues for migration, and signals for growth and differentiation. Integrins appear to be the primary mediators of cell-ECM adhesion, and they also serve as one of the many families of molecules active in cell-cell adhesion.

1.2.1 Structure of Integrins

Integrins are heterodimeric transmembrane glycoproteins consisting of non-covalently linked α and β subunits. Both α and β subunits contain a short carboxyl-terminal cytoplasmic domain, a single membrane-sparing region, and a large globular extracellular domain. As reviewed by Albelda and Buck (1990), the β subunits of all integrins are remarkably similar with a 40% to 48% amino acid sequence homology. There are four repeating units of cysteine residues in the extracellular domain. The first cysteine-rich repeat forms disulfide bonds with cysteines near the amino terminus to stabilize the folded structure of the extracellular domain of the β subunit. The amino acid homology between different α subunits is 20% to 25%. The extracellular domain of the α subunits contains calcium-binding regions. Some of the α subunits (*e.g.* α3, α5, α6) are composed of a heavy and light chain joined by a single disulfide bond near the transmembrane region. This gives the characteristic bands in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) after reduction (Larjava, 1991).

The amino-terminal globular domains from both α and β subunits form the extracellular ligand-binding regions whereas the cytoplasmic domains form connections with components of the cell cytoskeleton as well as with signal

transducing proteins. These enable the integrins to serve as a link between the cytoplasm and the ECM. Cytoplasmic receptors for integrin cytoplasmic domains have been identified. These include talin, α-actinin, calreticulin, β3-endonexin, and cytohesin-1 (reviewed in Hughes and Pfaff, 1998). The most extensively studied model for integrin-cytoskeletal transmembrane linkages is the adhesion plaque, or focal adhesion. A number of structural proteins (*e.g.* vinculin, talin and paxillin) and signaling proteins (*e.g.* tyrosine kinases, protein kinase C, and several phosphoproteins) have been identified in the adhesion plaques. The particular integrins that localize in focal adhesions are substrate-dependent. For example, the α5β1 fibronectin receptor, but not αvβ3 vitronectin receptor, localizes in focal adhesions on fibronectin substrates and vice versa (reviewed in Sastry and Horwitz, 1993).

The α subunits tend to associate exclusively with one of the β subunits with few exceptions. Accordingly, integrins are then divided into subfamilies on the basis of the β subunits. The β1 subfamily is widely expressed and is principally involved in cell-substrate interactions. The β2 subfamily is expressed on leukocytes and is involved in cell-cell interactions. The β3 subfamily is mostly expressed on platelets. The β4 subunit is mainly associated with α6 and is specifically located in the basement membrane zone, a part of hemidesmosomes, of the epidermis. However, some α subunits can associate with different β subunits, for examples, α6 and αv. The α6 subunit can bind with both β1 and β4, whereas αv can bind with β1, β3, β5 and β6 integrins. Figure 1-2 illustrates the subunit associations of human oral keratinocyte integrins. In normal keratinocytes, the complexes αvβ1 and αvβ3 are not found. The α6 subunits combine exclusively with the β4 localized in the hemidesmosomes.

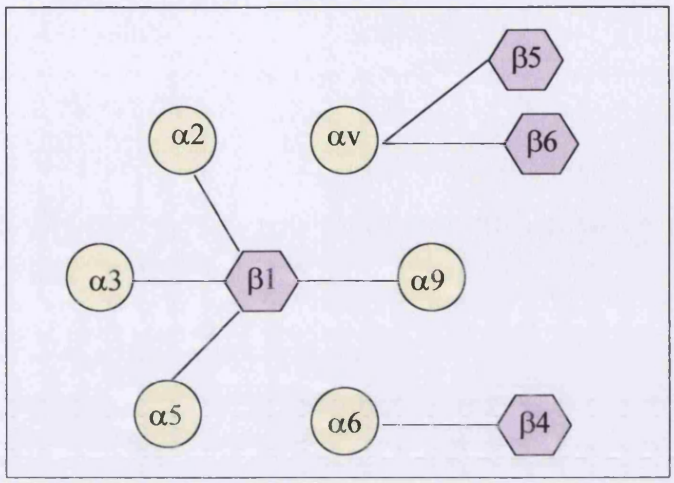


Figure 1-2 Schematic diagram showing the subunit associations of human oral keratinocyte integrins.

1.2.2 Integrin Ligands

Many integrins bind to ECM proteins and thereby mediate cell-ECM interactions. Examples of ECM ligands for integrins are fibronectin (FN), fibrinogen (FG), laminin (LM), various collagen types, entactin, tenascin, thrombospondin, von Willebrand factor, and vitronectin (VN) (reviewed in Hynes, 1992). Other integrins bind to cell membrane proteins, mediating direct cell-cell adhesion. These include intercellular adhesion molecule (ICAM)-1 and ICAM-2. Some ECM proteins, such as fibrinogen, can mediate cell-cell aggregation.

The recognition amino acid sequence for many of the integrins is the tripeptide arginine-glycine-aspartic acid or RGD. The RGD sequence is found within a number of ECM proteins including FN, FG, VN, LM, type I collagen and thrombospondin (Ruoslahti, 1991). The conformation of the RGD site appears to determine which integrin the RGD peptide or peptides will bind. Short synthetic peptides containing the RGD sequence can be designed to exhibit varying integrin specificities by restricting the conformation of the peptide through cyclization. However, not all integrins bind to RGD sequences in the ECM. For example, integrin $\alpha5\beta1$ binds the RGD sequence in FN but $\alpha4\beta1$ binds the leucine-aspartic acid-valine

(LDV) sequence in the alternative splicing type III region of FN. Keratinocytes use the collagen receptor α2β1 integrin to adhere to and migrate on collagens. However, collagen-driven motility is not mediated by the RGD although collagens contain these sequences (reviewed in Woodley, 1996).

1.2.3 Integrin Function

Integrins can transduce two forms of signals: inside-out and outside-in signaling. The former involves regulation of the affinity and/or specificity of integrin for its extracellular ligands. It has been hypothesized that structural alterations in the integrin cytoplasmic tails are propagated across the membrane to induce conformational changes at the ligand-binding site, and these conformational changes are associated with changes in binding affinity of the integrins (reviewed in Hynes, 1992; Sastry and Horwitz, 1993; Hughes and Pfaff, 1998). For outside-in signaling (reviewed in Clark and Brugge, 1995; Schlaepfer and Hunter, 1998), the events of integrin occupancy and clustering trigger a variety of cellular responses via signal-transduction proteins and the generation of second messengers. The cellular responses after integrin binding include actin polymerization and cell spreading, induction of gene expression, initiation of differentiation, and suppression of apoptosis.

Ligand binding to the β1 integrins generates at least two signals in keratinocytes (Levy *et al*, 2000). One signal results in the clustering of receptors into focal adhesions and polymerization of actin filaments, providing a positive stimulus for cell adhesion and spreading. The other signal is independent of receptor clustering in focal adhesions and cytoskeletal assembly and is a negative stimulus for differentiation.

1.2.4 Integrin-mediated Cell Adhesion

The extracellular domains of integrins mediate both cell-cell and cell-matrix interactions. The expression and the activity (affinity and avidity) of integrins can be regulated under various physiologic and pathologic conditions, resulting in a change in the adhesion properties of the cell. The affinity, reflected in the strength of ligand binding, can be changed directly by action of cations or ligands on the extracellular

domain, or artificially by activating antibodies. Alternatively, integrin affinity and avidity can be regulated from within the cell, by signals induced by receptor agonists (inside-out signaling process).

Cations have been shown to activate integrins by inducing a conformational change that results in the exposure of activation-dependent neoepitopes (Altieri, 1991; Dransfield *et al*, 1992). Ca^{2+}, Mg^{2+}, and Mn^{2+} have distinct effects on cell adhesion. Most cells prefer Mg^{2+} and Mn^{2+} over Ca^{2+}. It has been shown that the $\alpha5\beta1$ integrin contains at least three cation binding sites: a high affinity site for Mn^{2+}, a single low affinity binding site for both Mg^{2+} and Ca^{2+} and a high affinity binding site for Ca^{2+} (Mould *et al*, 1995). At low concentration, Ca^{2+} binds to the high affinity Ca^{2+} binding site and promotes Mg^{2+} binding to the integrin, inducing an increase in cell adhesion. In contrast, at high concentration, Ca^{2+} binds to the low-affinity Mg^{2+} binding site and inhibits adhesion. Studies on human epidermal keratinocytes have shown that Mg^{2+} alone enhanced the adhesion of the keratinocytes to collagen type I, laminin and fibronectin (Lange *et al*, 1994). The Mg^{2+}-enhanced adhesion was mediated by $\alpha2\beta1$ (on collagen type I), $\alpha2\beta1$ and $\alpha6\beta1$ (on laminin), and $\alpha5\beta1$ (on fibronectin) integrins. Interestingly, Ca^{2+} alone slightly enhanced the adhesion of the epidermal keratinocytes to fibronectin but not to collagen type I or laminin (Lange *et al*, 1994). At high concentration (>2 mM), Ca^{2+} suppressed the effect of Mg^{2+}-enhanced adhesion of keratinocytes to collagen type I and laminin but not to fibronectin.

1.2.5 Integrin-mediated Cell Migration

Cell migration requires a dynamic interaction between the cell, its substrate, and the cytoskeleton-associated motile apparatus. As reviewed by Huttenlocher *et al* (1995), initiation of cell locomotion involves a directional protrusion of the leading edge, presumably via actin polymerization, to form a lamellipodium, with its subsequent attachment to the substratum. Cell surface adhesion receptors serve to connect the substratum with the cytoskeleton. After formation and stabilization of the lamellipodium, cells use these adhesive interactions to generate the traction and force required for cell movement. The final step in the migratory cycle is the release of adhesions at the cell rear, with its subsequent detachment and retraction, allowing the

cell to advance over its substrate. Integrins play a central role in mediating cell migration, both as a structural link between the ECM and the actin cytoskeleton and as receptors that initiate signaling processes contributing to cell migration. It has been shown that maximal cell migration speed on fibronectin and type IV collagen occurred at an intermediate level of cell-substratum adhesiveness (DiMilla *et al*, 1993), indicating the importance of integrin receptors-substrate affinity in modulating migration rate (Huttenlocher *et al*, 1996). Cation-dependent affinity changes of integrins are responsible for cell adhesion to and de-adhesion from ECM proteins and have an effect on integrin-mediated cell migration. It has been shown that Mg^{2+} (10 mM) or Ca^{2+} (2 mM) alone induced keratinocyte migration on collagen type I and fibronectin but not on laminin (Lange *et al*, 1995). At higher concentrations, Ca^{2+} suppressed the stimulatory effect of Mg^{2+} on cell migration.

It has been shown that the migration of keratinocytes on substrate is mediated by α2β1 (on collagen type I), and α3β1 and α5β1 (on fibronectin) integrins (Lange *et al*, 1995). Integrin αvβ6 is a fibronectin receptor (Busk *et al*, 1992), which is not detectable in normal epithelium but is neo-expressed in epithelial wound healing (Haapasalmi *et al*, 1996) and oral squamous cell carcinomas (Jones *et al*, 1997). Increased expression of the αvβ6 integrin in oral squamous carcinoma cells has been shown to increase cell migration on fibronectin (Thomas *et al*, 2001a).

1.3 Integrin Expression in Human Epidermis and Oral Epithelium

Stratified squamous epithelium mainly expresses integrins of the β1 family, α6β4 and αv (Hertle *et al*, 1991; Jones *et al*, 1993). In normal epidermis, expression of each integrin (α2β1, α3β1, α5β1, α6β4, αvβ5) is mainly confined to the basal layer, but faint staining of the first 1 to 2 suprabasal layers is sometimes seen (Hertle *et al*, 1991). All of the integrins show a pericellular staining pattern with more intense staining along the lateral cell borders, but α6 and β4 are concentrated at the basement membrane zone. The strongest staining detected is for integrin subunits α2, α3, β1, α6 and β4. Integrin subunits α1, α5, and αv show either very weakly staining or absence (Klein *et al*, 1990; Hertle *et al*, 1991; Cavani *et al*, 1993). Integrin subunits α4 and β3 are never detected in the epidermis (Hertle *et al*, 1991; Cavani *et al*, 1993).

In normal oral epithelium, expression of the β1 and β4 families is similar to that seen in skin (Hormia *et al*, 1990; Jones *et al*, 1993). The α2, α3, α6, β1, β4 subunits are all highly expressed with weaker expression of α5 and αv (Jones *et al*, 1993). There is no expression of the β3 and β6 subunits in the epithelium of buccal mucosa (Jones *et al*, 1997). Recent studies have also reported that there is no expression of αvβ6 integrin in keratinized (gingiva, alveolar mucosa and palatal mucosa) and non-keratinized (buccal mucosa and ventral tongue) normal oral mucosae (Häkkinen *et al*, 2000a). Expression of all subunits is strongest in the basal layer with a pericellular distribution. Unlike skin, extensive suprabasal staining of the integrins may also be seen, particularly in the floor of the mouth and lateral border of the tongue (Jones *et al*, 1993). This may reflect the increased turnover rate in the oral epithelium. Integrin α9 subunit is localized at the basal cells and shows strongly staining against the basement membrane in keratinized epithelium of gingiva, alveolar and palatal mucosa (Häkkinen *et al*, 2000a). The α9 subunit is expressed more strongly in non-keratinized buccal mucosa and ventral tongue compared with normal keratinized gingiva with a pericellular distribution at the basal and immediately suprabasal cell layers (Häkkinen *et al*, 2000a). The α6 and β4 subunits are concentrated at the basement membrane zone. Integrins are largely absent from the suprabasal layers of terminally differentiating cells. Suprabasal expression of integrins can result in epithelial hyperproliferation (Jones *et al*, 1993; Rikimaru *et al*, 1997).

1.4 Cultured Human Keratinocytes

Like the surface epithelium, primary cultured keratinocyte sheets contain heterogeneous cell populations with different capacities for multiplication named holoclone, paraclone and meroclone (Barrandon and Green, 1987). Cells in holoclones have the highest proliferative capacity and probably represent stem cells. Paraclones contain cells which undergo terminal differentiation within a few cell generations as amplifying cells, and meroclones are intermediate between holocones and paraclones in their behaviour. The incidence of the different clonal types is affected by aging, since cells originating from the epidermis of older donors give rise to a lower proportion of holoclones and a higher proportion of paraclones.

Keratinocytes in culture show a similar integrin profile to those in *in vivo*. The integrin expression is largely confined to the basal layer and is decreased as the cells undergo terminal differentiation in the suprabasal layers (Adams and Watt, 1991). It has been reported that stem cells can be separated from amplifying cells or keratinocytes of lower proliferative potential on the basis of higher surface levels of the β1 integrins (Jones and Watt, 1993; Jones *et al*, 1995). In addition, terminally differentiating cells lack β1 integrins (Adams and Watt, 1990; 1991; Nicholson and Watt, 1991). Lack of the β1 integrin in terminally differentiating keratinocytes was shown to be due to the inhibition of gene transcription and intracellular transport by calnexin (Hotchin *et al*, 1995). The reduction in adhesiveness with the ECM precedes loss of the integrins (Adams and Watt, 1990).

1.4.1 Integrin Topography in Cultured Human Keratinocytes

Keratinocytes in culture conditions are considered to be in an activation stage associated with increased integrin expression.

α6β4 integrins

α6β4 integrins are detected on the basal surface of permeabilized cultured keratinocytes with a pattern of polymorphous patches, granular in appearance, and often suggesting a leopard skin pattern (Marchisio *et al*, 1991). They are not detected in the apical zones, or in association with talin at focal adhesions. Localization of the α6β4 at cell-cell contact areas and at the free edges of basal cells associated with cell-surface microvilli and filopodia in contact with the substratum has been observed in unpermeabilized keratinocytes grown on feeder cells (Adams and Watt, 1991).

β1 integrins

β1 integrins are expressed predominantly at the cell-cell contact areas of the basal keratinocytes (Adams and Watt, 1991; Marchisio *et al*, 1991), and are largely absent from the free margins of the basal cells (Nicholson and Watt, 1991). Using confocal laser microscopy, they can be detected a few micrometers upward from the plane of attachment within colonies, indicating that the β1 integrins are enriched on lateral surfaces (Marchisio *et al*, 1991). They are not detected in the cells that have

migrated outward and formed the upper layers of the colonies (Marchisio *et al*, 1991; Nicholson and Watt, 1991), whereas other junctional proteins *e.g.* vinculin (adhesion junction), cingulin (tight junction), desmoplakins 1 and 2 (desmosomes) are detected lining the boundaries among the tile-shaped cells of the upper layers (Marchisio *et al*, 1991).

α5β1 integrins

α5β1 integrins show varying expression on cultured keratinocytes. They are not detected in permeabilized keratinocytes (mAb P1D6, Marchisio *et al*, 1991), but expressed occasionally at cell-cell contact areas of unpermeabilized basal cells (mAbs BIIG2 and Mab16, Adams and Watt, 1991; Nicholson and Watt, 1991). Intense, localized staining at the free edges of some basal cells has been observed. Using immunoprecipitation with cell surface iodination, α5 subunits can be detected in keratinocytes from growing colonies, but not from confluent sheets of *in vitro* reconstituted stratified epithelium (Zambruno *et al*, 1995). However, the expression is much less abundant than the other β1 subgroups (α2β1 and α3β1).

αv integrins

Permeabilized keratinocytes express αv subunits mostly at the periphery of the basal aspect of expanding cells and in association with the endings of short microfilament bundles similar to vinculin and talin (Marchisio *et al*, 1991). This suggests that αvβ5 forms minute focal contacts in the basal keratinocytes. In unpermeabilized keratinocytes, αv subunits are detected as faint spotty staining of cell-cell contact areas between basal cells and, in some cells, intense staining of areas at the free margins of the cells (Adams and Watt, 1991).

β6 integrins

β6 integrin subunit is down-regulated in fully differentiated resting epithelia. The β6 subunits are absent in both normal human epidermis and normal oral mucosa (Breuss *et al*, 1995; Haapasalmi *et al*, 1996), although they are commonly expressed in squamous cell carcinomas derived from the oral mucosa (Jones *et al*, 1997). The β6 subunits (mAb R6G9) are not detected by immunofluorescent microscopy in

cultured epidermal keratinocytes with permeabilization, and the mRNA is also undetectable (Zambruno *et al*, 1995). On treatment with TGF-β1, the β6 subunits can be detected as continuous strands along intercellular boundaries and as tiny dots at focal contacts associated with the end of stress fibers (Zambruno *et al*, 1995). Using fluorescence-activated cell sorting analysis, it has been shown that freshly isolated epidermal keratinocytes do not stain for αvβ6 integrin (mAb E7P6) but begin to express this integrin after subculturing (Haapasalmi *et al*, 1996). Studies using oral squamous carcinoma cell line (VB6 cell line) grown on fibronectin have shown that the β6 (R6G9) subunits are prominently localized to the cell filopodia; αv (mAb L230) localized at cell-cell boundaries of the confluent cell monolayer (Thomas, 1999; Thomas *et al*, 2001b).

1.4.2 Integrin Ligands in Oral Keratinocytes

Oral keratinocyte integrins and their ligands are summarized below.

Integrin	*Ligand - Function*
α2β1	collagen and laminin, cell-cell adhesion (Carter *et al*, 1990)
α3β1	laminin, collagen, fibronectin, cell-cell adhesion (Carter *et al*, 1990; Adams and Watt, 1991)
α5β1	fibronectin (Adams and Watt, 1990; 1991)
α6β4 (hemidesmosome)	laminin (De Luca *et al*, 1990; Niesson *et al*, 1994)
α9β1	tenascin-C, fibronectin (Häkkinen *et al*, 2000a; Liao *et al*, 2002)
αvβ5	vitronectin (Adams and Watt, 1991; Busk *et al*, 1992)
αvβ6	fibronectin, tenascin, latent TGF-β1 (Busk *et al*, 1992; Huang *et al*, 1998; Munger *et al*, 1999; Thomas *et al*, 2002)

1.5 UP Keratinocytes

UP cells are a cell line of immortalized, but not tumourigenic human keratinocytes. They were derived from normal human epidermal keratinocytes transfected with the human papilloma virus (HPV)16 genome (Pei *et al*, 1991).

1.5.1 UP Cell Morphology and Growth Property

The morphology of UP cells differs from the parental cells. They are larger and flatter, and are less stratified even when they reach confluence. In the presence of a feeder layer, their growth rate is similar to that of the parental cells but they reach a higher confluent density and are immortalized. They can grow well in the absence of the 3T3 feeder cells (feeder independent) and have a reduced requirement for growth factors including hydrocortisone, insulin, cholera toxin and epidermal growth factor. UP cells can form colonies in soft agar (anchorage-independent growth), whereas parental cells cannot. However, the colony forming frequency is very low, approximately 0.1%, and colonies will not appear until 4-5 weeks after plating. The clonal growth potential of the UP cell line was shown to not relate to the level of HPV16 gene expression (Pei and Wu, 1993).

1.5.2 UP Cell Differentiation and Tumourigenic Potential

Similar to the normal parental cells, UP cells also contain abundant cytoplasmic keratin filaments and are joined by desmosome. However, the cells form fewer desmosomes than normal. Some highly vacuolated cells are present suggestive of necrosis. Involucrin, a marker for keratinocyte terminal differentiation, is expressed by UP cells in a lower proportion than the parental cells. UP cells do not form tumours in nude mice; they form cysts after injection as the parental cells do. However, the cysts are less well organized than those formed by their normal parental cells (Pei *et al*, 1991).

1.5.3 Integrin Expression and Function of UP Cells

Immunofluorescence, immunoprecipitation and flow cytometry have demonstrated that UP cells have lower levels of integrins ($\beta1$, $\beta2$, $\alpha2$, $\alpha3$, $\alpha5$, and $\alpha6$) than their parental cells (Hodivala *et al*, 1994). The integrin staining on cell

monolayers is heterogeneous, with some basal cells more intensely stained than others, and shows a concentration at cell-cell borders. The reduced expression of the $\alpha5\beta1$ and $\alpha2\beta1$ integrins on the UP cells is correlated with reduced adhesion to fibronectin and collagen. There are no significant differences between the normal and immortalized UP cells in adhesion to laminin. Reduced integrin expression has also been shown to relate to decreased motility on tissue culture plastic. Introduction of activated *ras* into UP cells has no effect on integrin levels, cell motility or tumourigenicity in nude mice, but increases adhesion to fibronectin (Hodivala *et al*, 1994).

It has been reported that UP cells, like normal oral keratinocytes (NHK), express matrix metalloproteinase (MMP)-2 and MMP-9. However, only MMP-9 is inducible in UP cells in response to scatter factor (SF). In NHK, both MMP-2 and MMP-9 are increased in response to SF (Bennett *et al*, 2000). Expression of class II molecules of the Major Histocompatibility Complex (MHC) *in vitro* can be induced on keratinocyte lines by the cytokine interferon, however, there is no expression by UP keratinocytes (Barrett *et al*, 2000).

1.6 Integrin Expression in Wound Healing

During wound repair, keratinocytes of the wound margin lose their contact with basement membrane and start migrating on a provisional matrix in the wound bed. This provisional matrix contains several proteins including FN, VN, tenascin and collagens (Larjava *et al*, 1996). *In vitro*, keratinocytes are able to attach to several ECM components such as FN, collagens, LM and VN. They also migrate well on FN and collagen but not on laminin (Nickoloff *et al*, 1988; Woodley *et al*, 1988). Several studies suggest that FN-receptors are nonfunctional in resting epidermis because freshly isolated keratinocytes do not attach on FN surfaces nor express the receptors. The expression is up-regulated when the cells are cultured for longer periods or are harvested from the wound (Grinnell, 1992a; Yamada *et al*, 1996a). This observation gives indirect evidence that there is little synthesis in the normal epidermis but that it could be up-regulated in situations like wound healing. The up-regulation of integrins may be essential for keratinocytes to migrate over the wound bed.

After wounding, keratinocytes switch to a migratory phenotype. Polarized integrin distribution in normal epithelium around the wound is lost. At a molecular level, the migratory phenotype is characterized by a diffuse distribution of β1 integrin receptors, little organized cytoskeleton, and generally poor organization of cell surface FN fibrils. The stationary phenotype is characterized by integrin organization into focal adhesion (FA) sites. These FAs are associated extracellularly with FN fibrils and intracellularly with specialized cytoskeletal complexes composed of talin, α-actinin, and paxillin into which actin bundles appear to insert (reviewed in Yamada *et al*, 1996a). Migrating keratinocytes do not express the classical basement membrane components such as type IV collagen and laminin-1, but deposit laminin-5 (Larjava *et al*, 1993; 1996). Distribution of α6β4 integrin changes from hemidesmosomal to diffuse during cell migration (Kurpakus *et al*, 1991).

Expression of β1 integrins is strongly up-regulated during wound healing (Cavani *et al*, 1993; Larjava *et al*, 1993). Migrating keratinocytes show increased expression of α5β1 integrins both *in vivo* (Clark, 1990; Cavani *et al*, 1993) and *in vitro* (Guo *et al*, 1991). However, Clark *et al* (1996) did not observe this higher expression of α5β1 integrin in migrating epidermis than in normal adjacent skin. Recently, Häkkinen *et al* (2000a) have demonstrated the up-regulation of α9 integrin subunit at the basal cell membrane of the migrating basal epithelial cells in early wounds. Its expression coincides with expression of tenascin-C under the migrating epithelial front. These data suggest that α9β1 integrin mediates cell migration on tenascin-C-containing provisional matrix and may regulate epithelial cell proliferation during wound re-epithelialization.

αv integrin subunit is only weakly expressed in normal oral mucosa (Jones *et al*, 1993) but expression is markedly increased during wound healing (Cavani *et al*, 1993; Larjava *et al*, 1993; Haapsalami *et al*, 1996). αvβ5 integrins have been detected in human excisional epidermal wounds (Clark *et al*, 1996), but not in mucosal wounds (Larjava *et al*, 1993; Haapasalmi *et al*, 1996). αvβ6 integrins are neo-expressed in the migrating basal keratinocytes during wound healing (Breuss *et al*, 1995; Clark *et al*, 1996; Haapasalmi *et al*, 1996). It has been demonstrated that the basal epidermal cells express αvβ5 during the first few days of migration (Clark

et al, 1996). In contrast, αvβ6 is expressed relatively late, around the time of epithelial fusion and starting differentiation (Clark *et al*, 1996; Häkkinen *et al*, 2000a). This progression of αv receptor expression corresponds to the early presence of VN, a ligand for αvβ5, and the later appearance of tenascin, a ligand for αvβ6. The early expression of αvβ5 is thus characteristic of a migratory phenotype but not of a differentiated and stationary phenotype. The late appearance of αvβ6 suggests that it is not necessary for keratinocyte migration but rather may provide proliferation signals to the healing epidermis from the underlying tenascin-rich provisional matrix (Clark *et al*, 1996; Haapasalmi *et al*, 1996). In addition, the ability of αvβ6 integrin to activate latent TGF-β1 (Munger *et al*, 1999) suggests that it may play a role in the activation of this growth factor to stimulate matrix production by fibroblasts in the wound granulation tissue (discussed in Häkkinen *et al*, 2000a).

1.7 *Staphylococcus aureus*

Staphylococcus aureus is considered an extracellular pyogenic pathogen. It is commonly found in the nasal cavity and on skin. Isolation of *S. aureus* from the mouth has been reported in approximately 24% of healthy adults and 64% of healthy children (Jackson *et al*, 1999). *S. aureus* causes several diseases in humans including skin infections, scalded-skin syndrome, toxic shock syndrome, infective endocarditis, septic arthritis, and osteomyelitis. It is a common infecting flora in simple wound infection (de Lalla, 1999; Giacometti *et al*, 2000). Many micro-organisms, including *S. aureus*, have been isolated (<10,000 cfu/mm^2 of ulcer surface) from skin ulcers showing no clinical signs of infection (Hansson *et al*, 1995). *S. aureus* is also implicated in some orofacial infections such as angular cheilitis (MacFarlane and Helnarska, 1976) and oral mucositis in elderly dehydrated patients (Bagg *et al*, 1995). Oral staphylococcal mucositis in patients with orofacial granulomatosis and inflammatory bowel disease has also been reported recently (Gibson *et al*, 2000). *S. aureus* in the oral cavity has been considered to be an important source of infection in septic arthritis (Jackson *et al*, 1999). How the oral mucosal resistance breaks down and develops mucositis; or how the bacteria breach the epithelial barrier to cause systemic complications is not yet well-understood.

1.7.1 General Characteristics of *Staphylococcus aureus*

Staphylococci are Gram-positive cocci of 0.5 to 1.5 μm diameter. They are arranged in characteristic grape-like clusters due to their ability to divide in many planes. Staphylococci are non-spore forming, non-motile, and usually catalase-positive (Samaranayake, 2002). They are usually non-encapsulated, or have limited capsule formation. Comparison of some characteristics of *S. aureus* from the less pathogenic *S. epidermidis* are listed in Table 1-1.

S. aureus grows aerobically as yellow or gold colonies (1 to 3 mm in diameter) on blood agar. Colonies of *S. aureus* that have developed for several days are usually large, smooth, slightly raised, and translucent. Almost all strains of *S. aureus* synthesize protein A which has a special affinity to the Fc fragment of

immunoglobulin G (IgG). A variety of enzymes and toxins are produced by *S. aureus*, although no one strain produces the whole range listed in Table 1-2.

Table 1-1 Characteristics of *Staphylococcus aureus* compared to *Staphylococcus epidermidis*.

	S. aureus	*S. epidermidis*
Catalase test	+	+
Coagulase test	+	-
Hemolysis on blood agar	+	±
Latex agglutination test	+	-
Mannitol fermentation test	Most strains	Few strains

Table 1-2 Toxins and enzymes produced by *Staphylococcus aureus*.

Toxin/Enzyme	Activity
Toxins	
Cytotoxin (α-, β-, γ-, δ-hemolysin)	Cell lysis
Leucocidin	Kills leukocytes
Epidermolysin (exfoliative toxin A, B)	Exfoliation and splitting of epidermis
Toxic shock syndrome toxin-1	Shock, rash, desquamation
Enterotoxin (A-E)	Induces vomiting and diarrhea
Enzymes	
Coagulase	Clots plasma
Catalase	Affects bactericidal activity of polymorphs
Elastase	Connective tissue breakdown
Hyaluronidase	Connective tissue breakdown
DNAase (nuclease)	DNA hydrolysis
Lipase	Breaks lipids of cell membrane
Penicillinase	Breaks down β-lactam drugs
Protein A	Antiphagocytic

1.7.2 Expression of Virulence Factors in Culture

A typical growth curve of a strain of *S. aureus* grown in nutrient medium is divided into the classic lag, log (exponential), post-exponential, and stationary phases of growth (Figure 1-3). Bacteria in lag phase would initiate the infection and would enter exponential phase, where multiplication would begin, accompanied by synthesis of surface proteins. Crowding during post-exponential phase would activate a density sensing mechanism that would stimulate toxic exoprotein production, enabling the organisms to escape from the localized infection (abscess) during the stationary phase and spread to new sites, where the cycle would be repeated. The virulence factors of *S. aureus* produced during different growth phases are listed in Table 1-3.

1.7.3 *S. aureus* in the Healthy Oral Cavity

The normal oral flora comprises a diverse group of micro-organisms including bacteria, fungi, protozoa and possibly even viruses. The presence of *Staphylococcus* species as a component of the normal oral flora is controversial. However, there are increasing data to suggest that staphylococci can be isolated frequently from the oral cavity of healthy individuals (reviewed in Smith *et al*, 2001). Most studies sample the oral cavity by use of mucosal swabs, mouthrinses or plaque scrapings. Oral carriage of *S. aureus* in healthy children has been reported ranging from 37% to 64% (Miyake *et al*, 1991; Jackson *et al*, 2000; Millar *et al*, 2001), of which 2% to 5% are methicillin-resistant *S. aureus* (MRSA). Oral carriage of *S. aureus* in healthy adults ranges from 24% to 36% (Jackson *et al*, 1999; Smith *et al*, 2001). In studies of denture-wearing individuals, carriage rates of *S. aureus* have been reported ranging from 23% to 48% (Dahlén *et al*, 1982; Tawara *et al*, 1996). Bacterial colonization of the upper respiratory tract has been reported to occur as early as the first months of life (Mackowiak, 1982). Recent study of the carriage rate of bacteria from tonsilar swabs of healthy infants (under 2-year of age) showed that 19.8% of cultures were positive (Berkovitch *et al*, 2002). In addition, the most common colonizing bacterium found in that study was *S. aureus* which represented 9.9 percent.

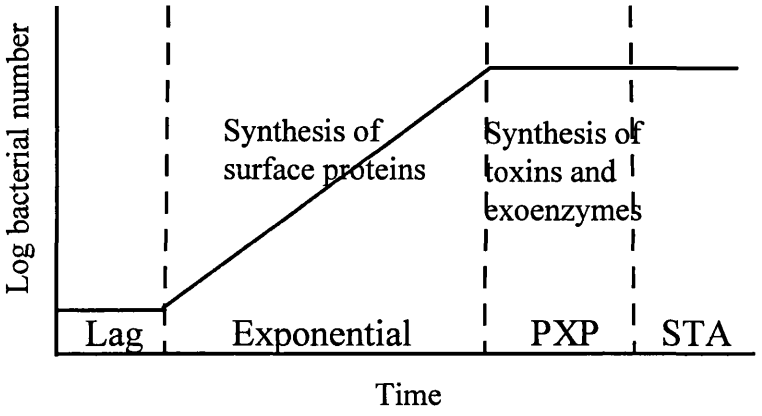


Figure 1-3 A typical growth curve of *Staphylococcus aureus* grown in nutrient medium. PXP, post-exponential phase; STA, stationary phase.

Table 1-3 Virulence factors of *S. aureus* during different growth phases.

Phase of growth	Virulence factor production
Log phase	Coagulase
	Clumping factor
	Fibrinogen-binding protein
	Fibronectin-binding proteins
	Protein A
Post-exponential	α-hemolysin
	β-hemolysin
	γ-hemolysin and PVL
	δ-hemolysin
	Enterotoxin B and C
	Exfoliative toxins A and B
	Hyaluronate lyase
	Lipase
	V8 Protease
	TSST-1
	Capsular polysaccharide
Throughout growth	Enterotoxin A

PVL, Panton-Valenine leukocidin; TSST-1, toxic shock syndrome toxin-1. [Modified from Projan SJ and Novick RP (1997). The molecular basis of pathogenicity. In: Crossley KB, Archer GL, eds. *The staphylococci in human disease*. Churchill Livingstone Inc: New York, pp. 55-80.]

1.7.4 *S. aureus* in Oral Diseases

Although *S. aureus* has a pathogenic potential, it is rarely associated with acute dento-alveolar infections. Oral infections from which *S. aureus* has been isolated include infected jaw cysts, oral mucosal lesions, denture-induced stomatitis, and angular cheilitis (reviewed in Smith *et al*, 2001). *S. aureus* is also responsible for the mucosal inflammation in orofacial granulomatosis and Crohn's disease (Gibson *et al*, 2000). *S. aureus* produces and secretes a number of surface proteins, enzymes and toxins that have been implicated as potential pathogenic factors (Projan and Novick, 1997; Dinges *et al*, 2000). It has been reported that 19% of *S. aureus* isolates from healthy children produce exfoliative toxin and 40% produce enterotoxin (Miyake *et al*, 1991). In addition, three of five patients with staphylococcal mucositis have been reported to be colonized by toxic shock syndrome toxin (TSST)-1 producing strains (Bagg *et al*, 1995). This suggests that heavy colonization of the oral cavity with toxin-producing strains may cause local mucosal damage.

More recently, a multicenter study on bacteriologic findings associated with chronic maxillary sinusitis in adults has demonstrated that *S. aureus* accounted for 10% of the isolated aerobic pathogens, although the most commonly isolated aerobes were *Streptococcus* species (21.4%) (Finegold *et al*, 2002). The *S. aureus* isolates had a high rate of resistance (50% to 67%) to penicillin G and ampicillin, but were highly susceptible (93%) to ciprofloxacin.

1.7.5 *S. aureus* in Wound Infection and Healing

Despite the use of prophylactic antibiotics and application of antiseptic principles to the practice of surgery, the association between staphylococci and postoperative wound infections remains strong. The frequencies of severe postoperative wound infections (fistulation and pus-formation) in patients undergoing major head and neck surgery involving direct wound communication between skin and mucosa of the oral cavity or the pharynx have been reported to be 23% to 25% (Friberg and Lundberg, 1990). The most frequently isolated pathogens in the wound infections was β-lactamase producing *S. aureus*.

Staphylococcal wound infections can cause a healing delay and may increase risk of death in immunocompromised patients. It was reported that complete healing of venous leg ulcers within 180 days was observed in only 21.6% of patients with *S. aureus*-infected ulcers compared with 62.5% of those without (Madsen *et al*, 1996). A study of diabetic patients with foot ulceration showed that 52% of patients with *S. aureus* died compared with 20% of patients whose foot ulcers were not infected with *S. aureus*, and the overall five year mortality rate was also increased (10.4% compared with 4%) (Mantey *et al*, 2000).

1.7.6 Staphylococcal Virulence Factors in Tissue Invasion

Most membrane- and tissue-damaging toxins (cytotoxins) are known as hemolysins because of their effect on rabbit, sheep and other animals erythrocytes. The most likely pathogenic role for these toxins could be the production of tissue damage and invasion of bacteria to form a localized infection. The toxin-associated tissue damage probably results from cellular disruption caused by formation of pores in cell membranes resulting in transmembrane fluxes of small molecules, and loss of cell integrity. Synthesis of exotoxins and enzymes predominates in the transition to the post-exponential phase of growth, while the expression of adhesion proteins is repressed (Proctor *et al*, 1982; Saravia-Otten *et al*, 1997). Two most studied cytotoxins produced by *S. aureus*, α- and β-toxin, are discussed.

Alpha-toxin (α-hemolysin)

Alpha-toxin is a pore-forming hemolytic toxin (Harshman *et al*, 1989) that causes membrane damage to many types of mammalian cells (reviewed in Bhakdi and Tranum-Jensen, 1991). It is particularly active against rabbit erythrocytes. Alpha-toxin is a 34 kDa protein consisting of 293 amino acid residues and lacking cysteine (Gray and Kehoe, 1984). The toxin monomers are secreted by *S. aureus* and bind to the membrane of a target cell, where they form cylindrical heptamers (Valeva *et al*, 1997) and subsequently an irreversible membrane-inserted pore (Valeva *et al*, 2001). It has been observed that α-toxin binds to rabbit erythrocytes via two different ways (Hildebrand *et al*, 1991). At low concentrations (<5 nM), the toxin binds exclusively via a high affinity interaction with an unidentified cellular receptor. At

high concentrations (>200 nM), the toxin will absorb nonspecifically to lipid bilayers resulting in permeabilization of the target cells. The size of the pore created by α-toxin exhibits slight variation (reviewed in Bhakdi *et al*, 1996). In planar lipid membranes and erythrocytes, the pore has been sized to 1-1.5 nm in diameter. In nucleated cells harboring the acceptor sites, however, the pores appear to be small, exhibiting a selectivity for monovalent ions. Pores formed in keratinocytes through application of low but lethal doses (≤ 1 μg/ml) of staphylococcal α-toxin appear considerably smaller than those formed in erythrocyte membranes (Walev *et al*, 1993). It has been reported that depletion of ATP by low-dose α-toxin in keratinocytes is irreversible (Walev *et al*, 1993), however, pore-forming defects and drop in cellular ATP can be repaired by fibroblasts (Walev *et al*, 1994; Valeva *et al*, 2000). The repair process is inhibited by cytochalasin D and inhibitors of lipid metabolism. Pore closure has been reported to be calcium and microtubule independent (Valeva *et al*, 2000).

The formation of pores that occur in non-erythrocyte cell lines may have cellular biological effects other than cell lysis. The very small pore produced by α-toxin in nucleated cells permits flux of only monovalent ions (Na^+, K^+). Depletion of K^+ ions leads to activation of interleukin-converting enzyme and massive release of mature IL-1β in cells containing the IL-1β precursor such as monocytes/macrophages (Walev *et al*, 1995). Secondly, T-lymphocytes that leak K^+ undergo apoptosis (Jonas *et al*, 1994). Alpha-toxin induced pore formation in endothelial cells has been shown to result in activation of arachidonic acid metabolism causing thromboxane and prostacyclin formation, increased vascular permeability and edema (Dinges *et al*, 2000). At a sublytic concentration, α-toxin (20 nM) has been shown to induce apoptosis in endothelial cells (Menzies *et al*, 2000). Exposure of α-toxin to keratinocytes has been reported to induce cytotoxic effects in a time and concentration dependent manner, and cells demonstrate the morphologic and functional characteristics of necrosis, not apoptosis, as observed by ethidium bromide/acridine orange assay (Ezepchuk *et al*, 1996). Addition of α-toxin to keratinocytes also induces release of tumour necrosis factor(TNF)-α into the medium within 30 min (Ezepchuk *et al*, 1996). Moreover, exposure of subcytocidal doses (40-160 ng/ml) of α-toxin to epithelial ECV340 (bladder carcinoma cell line) has been

reported to provoke a drop in cellular ATP level that is followed by activation of the transcription factor NF-κB and secretion of substantial amounts of IL-8 (Dragneva *et al*, 2001). These data suggest that formation of transmembrane pores by staphylococcal α-toxin can provoke a spectrum of events depending on target cell species and toxin doses. Repair of the lesions may follow in certain cases with partial ATP depletion.

Beta-toxin (β-hemolysin or Spingomyelinase C)

Beta-toxin acts as a Mg^{2+}-dependent sphingomyelinase and destroys membranes rich in sphingomyelin (Doery *et al*, 1963). Degradation of sphingomyelin by sphingomyelinase leads to generation of ceramide, which in turn is an intracellular messenger (Pushkareva *et al*, 1995). It is highly hemolytic for sheep but not rabbit red blood cells. Hemolytic activity of β-toxin is enhanced by incubation below 10°C after treatment at 37°C characterizing "hot-cold" hemolysis. Beta-toxin is a heat-labile exotoxin of 37 kDa when secreted into the culture medium.

Little is known about the effect of beta-toxin on cells other than erythrocytes. Beta-toxin degrades sphingomyelin in fibroblast membranes without affecting cell viability (Slotte and Bierman, 1988; Byers *et al*, 1992). Beta-toxin has been reported to selectively inhibit locomotion of human monocytes but not of granulocytes (Russell *et al*, 1975; Wilkinson, 1975). Walev *et al* (1996) have also reported a selective cytocidal effect of β-toxin from *S. aureus* for human monocytes (5 ng/ml) but not granulocytes, fibroblasts, lymphocytes and erythrocytes (5 μg/ml) within 60 min as determined by trypan blue stain and measurements of intracellular ATP. However, Marshall *et al* (2000) demonstrated that β-toxin did have a leukotoxic effect and that the leukotoxicity did not result from membrane lysis. Beta-toxin also induces release of IL-1β from monocytes, and this effect is probably associated with small permeability defects in monocyte membranes as observed with pore-forming α-toxin but not the effect mediated by ceramide (Walev *et al*, 1996). Using ^{3}H-thymidine assays, purified β-toxin as high as 46 μg/ml has been shown to have no effect on the proliferation of bovine mammary epithelial (MAC-T) cells (Matthews *et al*, 1994). In contrast, Cifrian *et al* (1996a) reported a cytotoxic effect of β-toxin on

MAC-T cells, and an additive effect of the β-toxin with α-toxin causing cell death as determined by propidium iodide incorporation when used at 32 hemolytic units per 100 μl.

1.7.7 *S. aureus* as an Intracellular Pathogen

There is increasing evidence that *S. aureus* can be internalized by non-phagocytic human cells *in vitro*. These include keratinocytes (Nuzzo *et al*, 2000; Jung *et al*, 2001), airway epithelial cells (Kahl *et al*, 2000), corneal epithelial cells (Jett and Gilmore, 2002), endothelial cells (Yao *et al*, 1995; Menzies and Kourteva, 1998), and osteoblasts (Jevon *et al*, 1999). Recently, the presence of intracellular living *S. aureus in vivo* has been reported in alveolar cells in milk samples of bovine mastitis (Hébert *et al*, 2000).

1.7.8 Factors Influencing *S. aureus* Internalization

Internalization of *S. aureus* in mammalian cells has been demonstrated to require active participation of cytoskeletal elements from host cells, principally actin. Inhibition of actin microfilament polymerization with cytochalasin D has been shown to decrease the number of internalized *S. aureus* in the human tracheal epithelial cell line CFT-1 (Kahl *et al*, 2000), osteoblast cell line MG-63 (Jevon *et al*, 1999) and corneal epithelial cells (Jett and Gilmore, 2002) by more than 90% compared with control. Depolarization of microtubules by colchicine decreases the number of internalized *S. aureus* in MG-63 and CFT-1 cells by only 40% and 66% respectively.

Host cell de novo protein synthesis has been reported to be required for the uptake of *S. aureus* in some cell types. Inhibition of protein synthesis with cycloheximide has been shown to decrease the number of internalized *S. aureus* in MG-63 cells (71 μM) by 35% (Jevon *et al*, 1999) but not CFT-1 cells (20 μg/ml) (Kahl *et al*, 2000). The use of chloramphenicol has shown that bacterial de novo protein synthesis is not required for efficient internalization (Jevon *et al*, 1999). In addition, formalin-fixed (Sinha *et al*, 1999) and heat-killed (Kahl *et al*, 2000) *S. aureus* are also effectively internalized. These indicate that internalization is not dependent on metabolically active organisms.

Signal transduction via a host-cell tyrosine kinase appears necessary for staphylococcal internalization. An inhibitor of protein tyrosine kinase, genistein, reduced MAC-T cell (Dziewanowska *et al*, 1999) and human corneal epithelial cell (Jett and Gilmore, 2002) uptake of *S. aureus* by 95% and >99.9% respectively compared with untreated controls. A recent study of intracellular signaling mechanisms has demonstrated the involvement of mitogen-activated protein kinase (MAPK) pathways in normal osteoblasts in response to *S. aureus* infection (Ellington *et al*, 2001). Adherence of *S. aureus* to normal osteoblasts has been shown to activate extracellular-signal-regulated kinase (ERK)-1 and -2 phosphorylation. The phosphorylation levels were much lower for UV-killed than live *S. aureus* cells.

Fibronectin, host cell integrin $\alpha 5\beta 1$ and *S. aureus* surface adhesins particularly fibronectin-binding proteins are considered to be essential in *S. aureus* infections in human. These are discussed in Sections 1.8 and 1.9.

1.7.9 Fate of Internalized *S. aureus*

S. aureus is able to internalize and survive in many mammalian cells. Examination using transmission electron microscopy has demonstrated the internalization of *S. aureus* into endosomes from which they subsequently escape (Bayles *et al*, 1998; Kahl *et al*, 2000). It has been reported that some vacuoles containing heat-killed but not live *S. aureus* are found to fuse with lysosomes (Kahl *et al*, 2000). Experiments using human airway epithelial cells CFT-1 have demonstrated the ability of internalized *S. aureus* to replicate intracellularly (Kahl *et al*, 2000). Recently, Qazi *et al* (2001) have shown that the accessory gene regulator (*agr*) induction occurs prior to endosomal lysis and that the production of *agr*-regulated exoproteins, such as hemolysins, appear to be required prior to the release and replication of *S. aureus* within the infected cells.

1.7.10 Consequences of *S. aureus* Internalization

It is now believed that intracellular replication plays an important role in the frequency and persistence of invasive staphylococcal infections, perhaps by providing protection against both host defenses and antibiotic treatments. Two main

consequences of intracellular *S. aureus* have been investigated including apoptosis and cytokine induction.

Internalized *S. aureus* has been shown to induce apoptosis in many cell types including endothelial cells (Menzies and Kourteva, 1998), keratinocytes (Nuzzo *et al*, 2000), airway epithelial cell line (Kahl *et al*, 2000), and bovine mammary epithelial cell line (Bayles *et al*, 1998). It has been shown that internalization of metabolically active *S. aureus* is necessary for apoptosis to occur (Menzies and Kourteva, 1998; Wesson *et al*, 1998; Kahl *et al*, 2000). Studies using an α-toxin deficient mutant have demonstrated the involvement of this toxin in endothelial cell apoptosis after ingestion of *S. aureus* (Menzies and Kourteva, 2000). The mechanisms of apoptosis have been reported to involve caspase 8 and 3 (Wesson *et al*, 2000), and TNF-related apoptosis-inducing ligand (Alexander *et al*, 2001).

Induction of cytokines after *S. aureus* internalization has been demonstrated in endothelial cells (Yao *et al*, 1995), epithelial cells (Wesson *et al*, 2000), and osteoblasts (Bost *et al*, 1999). Internalization of *S. aureus* by endothelial cells has been shown to result in the production of IL-6 and IL-1β (Yao *et al*, 1995). In epithelial cells (MAC-T), *S. aureus* internalization induces expression of TNF-α, IL-1β, IL-6, but not IL-8 or TGF-β (Wesson *et al*, 2000). The production of cytokines IL-6 and IL-12 have been reported to be induced in osteoblasts (Bost *et al*, 1999). Viable *S. aureus* is required to induce cytokine expression (Yao *et al*, 1995; Wesson *et al*, 2000).

The expression of surface molecules of human umbilical vein endothelial cells (HUVEC) after *S. aureus* internalization has been investigated. Beekhuizen *et al* (1997) reported that *S. aureus*-infected HUVEC displayed increased surface expression of VCAM-1, ICAM-1 and MHC I, but not P-selectin, E-selectin, PECAM-1 and ICAM-2 molecules. In addition, these infected HUVEC expressed hyperadhesiveness for monocytes and granulocytes recruiting the inflammatory cells to the site of infection. Recent studies using clinical isolates of *S. aureus* have shown that up-regulation of the adhesion molecules, E-selectin and ICAM-1, in HUVEC

varies between isolates (Strindhall *et al*, 2002). Some isolates induce a significant expression of E-selectin without stimulation of ICAM-1.

1.8 Staphylococcal Fibronectin-binding Proteins (FnBPs)

Adherence of bacteria to host tissues is the initial step in the process of infection and the first step for intracellular invasion. *Staphylococcus aureus* expresses an array of cell surface adhesins termed microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) that bind to components of ECM. The MSCRAMMs from *S. aureus* include FN-binding proteins (FnBPA and FnBPB), fibrinogen-binding proteins or clumping factors (ClfA and ClfB), collagen-binding protein (Cna), and elastin-binding protein. *S. aureus* FnBPs are likely the most important MSCRAMMs in internalization of *S. aureus* into eukaryotic cells.

In general, but not always, *S. aureus* has two closely linked *fnbA* and *fnbB* genes, encoding surface proteins designated FnBPA (110 kDa protein) and FnBPB (98 kDa protein) respectively (Signäs *et al*, 1989; Jönsson *et al*, 1991). Figure 1-4 illustrates the structural organization of FnBPA and FnBPB from *S. aureus*.

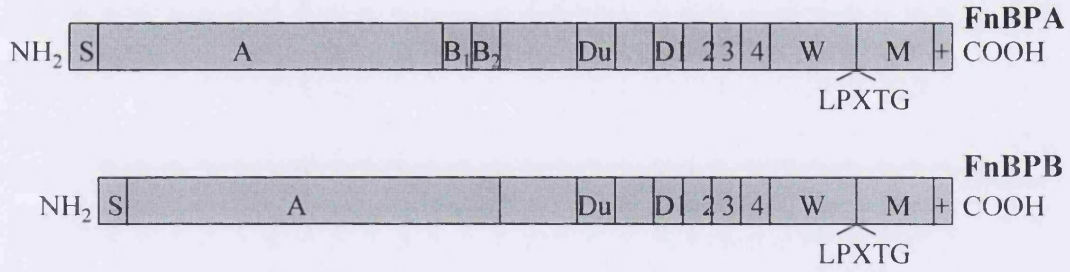


Figure 1-4 Structural organization of FnBPA and FnBPB of *S. aureus* strain 8325-4. S, amino-terminal signal sequence; A, fibrinogen-binding region (only 45% similar between FnBPA and FnBPB); B, almost identical repeats of 35 amino acid residues with unknown function, that is present in FnBPA but not in FnBPB; Du-D4, fibronectin-binding repeat units (~94% amino acid identity between FnBPA and FnBPB); W, cell wall-spanning region; M, cytoplasmic membrane-spanning region; +, positively-charged intracellular residues. The cell wall-anchoring LPXTG (Leu-Pro-X-Thr-Gly) motif is indicated between regions W and M.

Both *S. aureus* FnBPA and FnBPB are similar in their structural organization and mechanism of ligand recognition to the FN-binding proteins from other gram-positive cocci such as *Streptococcus pyogenes* and *Streptococcus dysgalactiae* (reviewed in Joh *et al*, 1999). A signal sequence involved in transport of the proteins through the cytoplasmic membrane is found at the amino terminus. The carboxyl terminus is composed of a conserved LPXTG (Leu-Pro-X-Thr-Gly) motif, a hydrophobic membrane-spanning region (M region), and a tail of positively-charged residues. The LPXTG motif is required for accurate sorting and anchoring of the proteins to the cell wall peptidoglycan (reviewed in Navarre and Schneewind, 1999). The FnBPA contains a unique N-terminal A region that is interrupted by two copies of a 35 amino acid repeat, termed B1 and B2. The FnBPB does not possess B repeats and is only 45% similar to FnBPA in the N-terminal domain. The N-terminal A regions of the FnBPs have been recently shown to bind specifically to fibrinogen (Wann *et al*, 2000).

The FN-binding activity of the FnBPs has been localized to the ~40 amino acid residue long repeat unit (D repeat) in the C-terminal portion of these proteins. FnBPA and FnBPB of *S. aureus* 8325-4 contain four tandemly repeat units (D1-D4) and a fifth repeat (Du) is located approximately 100 amino acid residues N-terminal to D1. The D4 is an incomplete repeat unit. These repeats are highly conserved (~94% amino acid identity) between the two proteins (reviewed in Fowler *et al*, 2000). The ability to bind FN is located exclusively within the C-terminal half of each D unit (McGavin *et al*, 1991; 1993; Huff *et al*, 1994). A recombinant protein of the D1-D3 repeats of FnBPA has been shown to inhibit the binding of both whole FN and the N-terminal fragment of FN to *Staphylococcus aureus*, *Streptococcus dysgalactiae*, and *Streptococcus pyogenes* (Joh *et al*, 1994). Recently, a second ligand-binding domain located outside the D repeat units has been reported and was identified to locate in the A region of FnBPA (Joh *et al*, 1998; Williams *et al*, 2002) and FnBPB (Williams *et al*, 2002). Identification of the binding site for the A region of FnBPA and FnBPB in FN using phage display of fragments of the proteins showed that both fragments bound to immobilized intact FN and the N-terminal 30 kDa FN, but not the 120 kDa FN fragment (Williams *et al*, 2002). A recombinant protein of the Du-D4 repeat units of FnBPA has recently been reported to interact

with heat shock protein 60 (Hsp60) from epithelial cells membrane including MAC-T, HEp-2 and Caco-2 cells (Dziewanowska *et al*, 2000).

The primary binding site in FN for the repeat units has been mapped to the first five Type I modules in the N-terminus (Mosher and Procter, 1980; Sottile *et al*, 1991). Deletion of any single Type I module of the fibronectin in constructed recombinants resulted in loss of binding to *S. aureus* (Sottile *et al*, 1991), indicating that all five Type I modules contribute to a single binding unit. Analysis of the interaction of N-terminal five Type I modules of FN with synthetic peptides of D repeat units, using fluorescence polarization and affinity chromatography, showed that the FN Type I module pair 4-5 (FNI_{4-5}) was the dominant binding site for the D3 unit (Huff *et al*, 1994). Further study using glutathione S-transferase (GST) fusion proteins showed that the Du-D4 fragment and each of the full-length D units (Du, D1, D2, D3) recognized the same FNI_{4-5} pair (Joh *et al*, 1998). A second bacterial-binding site in FN has been located in the C-terminal heparin-binding domain that binds to the staphylococcal and streptococcal MSCRAMMs, but comparatively weakly (Kuusela *et al*, 1984; Bozzini *et al*, 1992). The binding site for this domain appears to lie outside the repeat units in these proteins (Bozzini *et al*, 1992).

1.8.1 The Role of *S. aureus* FnBPs in the Internalization of *S. aureus* by Mammalian Cells

The interaction between *S. aureus* and host cells has been proposed to occur through a bridging model where FN is bound by *S. aureus* FnBPs as well as host cell $\alpha5\beta1$ integrin. Table 1-4 summarizes the supportive data from many cell types using either FnBP-deficient mutants, recombinant proteins of the FN-binding domain (rFnBP) or anti-integrin antibodies on bacterial internalization.

Table 1-4 Summary of the blocking function of *S. aureus* FnBPs and anti-integrin antibodies on *S. aureus* internalization.

	FnBP⁻ mutant	*rFnBP*	*anti-α5β1*	*anti-β1*
MAC-T	Yes[1]	D3, 10 μM (50%)[1] 20 μM (80%)[1] 100 μg coating[2]		No[1]
HUVEC	Yes[4]	D1-D4, 10 μg[3] CD region, 0.1 μM[4]	10 μg[4]	
GD25β1A[5]	Yes	D1-D3, 1 μM		β1-mutant
MG-63[6]	Yes	D1-D4, 10 μg	2 μg	
Epith293[7]	Yes	D1-D4, 10 μg	1:1000	
HSF[7]	Yes	D1-D4, 10 μg		
HEp-2[1]	Yes			1:200
HCEC[8]	Yes			
HaCaT[9]	Yes			

MAC-T, bovine mammary epithelial cell; HUVEC, human umbilical vein endothelial cell; GD25β1A, mouse fibroblast cell line; MG-63, human osteosarcoma cell line; Epith293, embryonic kidney cell line; HSF, human skin fibroblast; HCEC, human corneal epithelial cell; HaCaT, spontaneously immortalized skin keratinocyte line.

(1) Dziewanowska *et al*, 1999; (2) Dziewanowska *et al*, 2000;

(3) Menzies *et al*, 2002; (4) Massey *et al*, 2001; (5) Fowler *et al*, 2000;

(6) Nair SP (personal communication); (7) Sinha *et al*, 1999; (8) Jett and Gilmore, 2002; (9) Mempel *et al*, 2002.

Studies comparing internalization of FnBP-deficient mutants and the isogenic parental strains have demonstrated that FnBPs are essential for the internalization of *S. aureus* by human corneal epithelial cells (Jett and Gilmore, 2002), human keratinocyte cell line HaCaT (Mempel *et al*, 2002), bovine mammary epithelial cells (MAC-T) (Dziewanowska *et al*, 1999), human endothelial cells (Peacock *et al*, 1999), human embryonic kidney cell line (epithelial 293 cells) (Sinha *et al*, 1999), human osteoblast MG-63 (Ahmed *et al*, 2001), and mouse fibroblasts (Fowler *et al*, 2000). In addition, restored expression of *S. aureus* FnBPs in deficient mutants (Fowler *et al*, 2000) or construction of *S. aureus* FnBP expression into noninvasive strains such as *Staphylococcus carnosus* and *Lactococcus lactis* (Sinha *et al*, 2000; Massey *et al*, 2001) restores the adhesive and invasive properties to these strains.

Many studies have demonstrated the inhibitory activity of recombinant FnBP proteins in the internalization of *S. aureus* by mammalian cells. These include MAC-T cells (Dziewanowska *et al*, 1999; 2000), mouse fibroblasts (Fowler *et al*, 2000), human skin fibroblasts, spontaneously transformed human umbilical vein endothelial cells and epithelial 293 cells (Sinha *et al*, 1999). The recombinant protein derived from the ligand-binding domain of *Streptococcus dysgalactiae* (rFNBD-B) which was shown to inhibit the binding of FN to *S. aureus* (Joh *et al*, 1994), also inhibited the adherence of *S. aureus* to endothelial cells (Peacock *et al*, 1999). A study comparing the inhibitory potency of the recombinant proteins has shown that a recombinant protein of the D1-D3 repeats of FnBPA (rFNBD-D) inhibited the internalization of *S. aureus* by mouse fibroblasts, GD25β1A, less effectively than the full-length protein, rFnBPA(37-881) (Fowler *et al*, 2000). A recombinant protein of the A region of FnBPA (including two B repeats) was an equally potent inhibitor of cellular invasion as rFNBD-D.

Decreased internalization of *S. aureus* has been reported in the presence of antibodies against integrin α5β1 (mAb JBS5) in endothelial cells (Massey *et al*, 2001) and epithelial 293 cells (Sinha *et al*, 1999). Interestingly, by using antibodies against the integrin β1 subunit (mAb P4C10), a dramatic reduction in the internalization of *S. aureus* by HEp-2 but not MAC-T cells has been observed (Dziewanowska *et al*, 2000). Studies using a β1 integrin-deficient mouse fibroblast

cell line (GD25) demonstrated reduction of internalized *S. aureus* by approximately 97% compared with the complemented cell line GD25β1A (Fowler *et al*, 2000). In addition, inhibition of *S. aureus* internalization by GD25β1A cell line was also observed in the presence of an RGD-containing peptide (Fowler *et al*, 2000). By using various anti-α5β1 antibodies with different ligand affinity to coat *S. aureus*, it was found that integrin-mediated internalization following bacterial attachment to the cell was primarily dependent on the affinity of the bacterial ligand for the integrin (Tran Van Nhieu and Isberg, 1993). Mutations of the cytoplasmic domains of β1-integrins at cyto-1 and cyto-2 have been demonstrated to eliminate internalization of *S. aureus* by HEp-2 cells (Tran Van Nhieu *et al*, 1996). These findings suggest that internalization of *S. aureus* occurs by receptor-mediated endocytosis, where FnBP-FN serves as the high affinity ligand for the β1-integrin host cell receptor.

Decreased internalization of *S. aureus* has been reported in the presence of antibodies against FN in endothelial cells (Dziewanowska *et al*, 2000), GD25β1A (Fowler *et al*, 2000) and epithelial 293 cells (Sinha *et al*, 1999). Alternatively, the presence of small amounts of exogenous FN have been shown to enhance internalization of *S. aureus* in the cells that lack endogenous FN expression such as HEp-2 cells (Dziewanowska *et al*, 2000) or cell suspension treated by trypsin/EDTA to reduce the amount of cell-associated FN (Sinha *et al*, 1999).

Hsp60 has recently been reported to be the host cell receptor for MAC-T cells to bind to *S. aureus* FnBP (Dziewanowska *et al*, 2000). The reaction of *S. aureus* FnBP with Hsp60 is essential for the most efficient internalization of *S. aureus* by MAC-T cells. However, Hsp60 is not required for internalization of *S. aureus* by endothelial cells (Massey *et al*, 2001).

1.8.2 Effects of *S. aureus* FnBPs on Cell Functions

S. aureus FN receptor (FnBP) acts as a multivalent receptor and exhibits a high affinity for the 29-kDa amino-terminal fibronectin fragment (FN-N29-kDa). It is also necessary for the bacterial internalization process through the FN bridge and host cell β1-integrin. However, studies on the effect of FnBP or FnBP-FN complex on host cells are limited. Miyamoto *et al* (2001) examined the effect of *S. aureus*

FnBPs on the activation and adhesion of human T lymphocytes using recombinant proteins of *S. aureus* FnBPA. It was found that immobilized recombinant truncated forms of the FnBPA (full-length rFnBPA(37-881), AB regions, and Du-D4 repeats) mediated T lymphocyte proliferation when co-immobilized with anti-CD3 monoclonal antibody (OKT3). The coactivation signal generated by the rFnBPA proteins required the presence of FN such as medium containing serum with FN or preloaded with soluble FN. Monoclonal antibody blocking studies revealed that integrin $\alpha5\beta1$ was the major receptor responsible for the rFnBPA costimulatory signal. Using a shear flow cell detachment assay, the rFnBPA proteins (full-length rFnBPA(37-881) and Du-D4 repeats) supported the FN-mediated adhesion of lymphocytes. These findings suggest that the complex of FnBP-FN is sufficient to mediate cell adhesion as well as generate a functional T cell costimulatory signal through integrin $\alpha5\beta1$.

A FN-binding receptor of *Streptococcus pyogenes*, termed protein F1, has a similar structural organization to the FnBPs of *S. aureus*, including C-terminal FN-binding repeats that are homologous to the D repeats of the FnBPs (Ozeri *et al*, 1996). Recently, the effect of recombinant proteins, derived from the ligand-binding domain of *Streptococcus pyogenes* protein F1, on FN matrix assembly and cell functions was investigated (Tomasini-Johansson *et al*, 2001). It was found that the functional upstream domain (FUD, 49-amino-acid residues), which binds to the Type I modules especially the N-terminal Type I modules of FN (Ensenberger *et al*, 2001), inhibited FN assembly by human skin fibroblasts. The FUD also inhibited the binding of FN or FN-N70-kDa fragment to fibroblasts plated on FN, or MG-63 osteosarcoma cells plated on vitronectin. Despite inhibition of FN assembly, the FUD had no effects on fibroblast adhesion, growth (determined by MTS assay), or focal contact or stress fiber formation.

Streptococcus pyogenes uses two different types of FN-binding receptors, protein F and protein M, to efficiently bind and internalize the host cells. In contrast, *S. aureus* appears to be solely dependent on FnBPs to mediate efficient internalization. It would be of interest to investigate whether FnBPs from *S. aureus*

affect FN assembly and cell functions in a similar or different way to the FN-binding protein from *Streptococcus pyogenes*.

1.9 Fibronectin

Fibronectin (FN) is a key component of the provisional matrix during wound repair (Yamada and Clark, 1996b), and it is often elevated during tissue remodeling and fibrosis and in neoplasia. Major functions of FN include mediating cellular adhesion, promoting cell migration and monocyte chemotaxis, and helping to regulate cell proliferation and gene expression. Fibronectin is a high molecular weight glycoprotein that exists in both soluble and insoluble forms. Soluble FN is found in micromolar concentration in many physiological fluids such as plasma, amniotic fluid, cerebrospinal fluid, bronchoalveolar lavage fluid, synovial fluid and pleural effusions. It is composed of two disulfide-bonded 210-250 kDa subunit polypeptides, forming a disulfide linked dimer. Circulating FN does not self-polymerize in physiologically relevant solutions, and there is little passive accumulation of FN in pre-existing ECM (reviewed in Magnusson and Mosher, 1998). Assembly of FN takes place at specialized areas on the cell surface. Insoluble FN (cellular fibronectin) is a dimeric or multimeric form made by certain cells in culture, resulting in a fibrillar matrix deposited on the cell surface and in the extracellular matrix.

1.9.1 Fibronectin Polypeptide

The FN subunit polypeptide is composed of a series of independently folding modular domains known as FN repeats I, II and III consisting of 12, 2 and 15-17 copies respectively (Figure 1-5). Type I repeats contain 45-50 amino acid residues, held together by two disulfide bonds. Type I repeats are confined to the carboxyl- and amino-terminal regions of the molecule. Type II repeats contain 60 amino acid residues with two disulfide bonds. Two of these segments interrupt a sequence of nine Type I segments at the amino-terminus to form the collagen-binding domain. The remaining central region of the molecule is composed of an uninterrupted sequence of 15-17 Type III repeats, each about 90 amino acid residues long and lacking disulfide bonds. The basic FN molecule is normally composed of two such

polypeptide chains covalently bound through two disulfide bonds, located near the carboxyl-terminus, forming a dimer of M_r 450000.

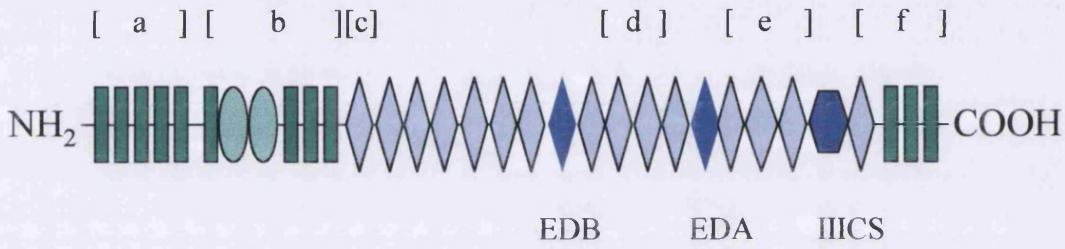


Figure 1-5 Domain structure of fibronectin. The fibronectin dimer is formed through interchain disulfide bonds at the carboxyl-terminus. Each subunit consists of 12 Type I (rectangles), 2 Type II (ovals), and 15-17 Type III (rhombuses) repeating modules. Sets of repeats make up domains for interacting with cells, other matrix components, and fibronectin itself.
[a], binding domain for *S. aureus*, heparin I, fibrin, fibronectin matrix assembly sites;
[b], binding domain for collagen (gelatin);
[c], matrix assembly site;
[d], major binding domain for cell at Arg-Gly-Asp (RGD) sequence in repeat III_{10} and synergy site in repeat III_9;
[e], binding domain for heparin II (proteoglycans);
[f], binding domain for fibrin.
EDA, EDB and IIICS are alternative splicing regions of the fibronectin mRNA. (modified from Schwarzbauer JE and Sechler JL (1999). *Curr Opin Cell Biol* 11: 622-627).

EDA (ED for "extradomain"), EDB and Type III connecting segment (IIICS) are alternative splicing regions of the FN mRNA. EDA and EDB regions are Type III repeat domains in human FN, which can be included or omitted from the mature mRNA. IIICS is located towards the carboxyl-terminus and corresponds to a domain of 120 amino acid residues. The CS1 region within the IIICS contains Leu-Asp-Val

(LDV) sequence which is the ligand for integrin α4β1. Fibronectin can vary structurally by alternative splicing of these three regions.

Alternative splicing of both the EDA and EDB exons is cell-specific. Hepatocytes only synthesize FN lacking both ED domains, while fibroblasts and many cell types produce all possible variants. Thus, plasma FN which is synthesized in the liver by hepatocytes contains neither EDA nor EDB, whereas cellular FN contains variable amounts of either or both EDA and EDB. Given the tissue-specific patterns of splicing, it seems very likely that different splice variants have specific cellular effects. The EDA domain has been shown to be essential for chondrogenesis at the level of cellular condensation (Gehris *et al*, 1997).

1.9.2 Fibronectin Matrix Assembly

Fibronectin fibrillogenesis is not spontaneous and generally occurs only in the presence of cells. Adherent cells polymerize an insoluble FN matrix by assembling cell- or plasma-derived soluble FN into insoluble fibrils (Mosher *et al*, 1992). Integrin α5β1 on the cell surface is the major receptor responsible for FN matrix assembly (Ruoslahti, 1991; Wu *et al*, 1993). Another FN-binding integrin involved in FN assembly is αvβ3 (Wennerberg *et al*, 1996; Wu *et al*, 1996). However, a number of FN-binding integrins do not initiate FN assembly such as αvβ6 (Busk *et al*, 1992), αvβ1 (Zhang *et al*, 1993), and α4β1 (Wu *et al*, 1995). As a dimeric ligand, FN induces receptor clustering by binding to two integrins. Integrin clustering induces actin cytoskeletal rearrangements, resulting in a dramatic change in cell shape and increased tension. It has been shown that FN assembly is enhanced under conditions that activate Rho (Zhang *et al*, 1997; Zhong *et al*, 1998). Cell-derived tension, generated by Rho-induced cytoskeletal rearrangements, is necessary for FN matrix assembly in which it stretches cell-associated FN to unfold and expose the matrix assembly sites necessary for FN interactions during fibril elongation. It is believed that certain segments of FN that are involved in the assembly of FN fibrils need to be exposed by interactions with integrins, explaining why cells are needed for matrix assembly. Furthermore, FN matrix assembly, but not cell adhesion, is essential for proper stress fiber organization (Bourdoulous *et al*, 1998). It was shown that human umbilical vein endothelial cells plated on a mixture of laminin and FN

and treated with antibodies to both α5 (P1D6) and αvβ3 (LM609) integrins underwent reduced actin stress fiber organization and displayed filopodial protrusions at the cell periphery (Bourdoulous *et al*, 1998).

1.9.3 Effects of Fibronectin Matrix on Cell Functions

Fibronectin-rich matrices provide substrates for cell adhesion and migration during development, wound healing, as well as affecting many cellular functions including proliferation and differentiation. Cell interactions with FN activate a number of intracellular signal transduction cascades. In particular, phosphorylation of focal adhesion kinase ($pp125^{FAK}$) is an early biochemical response to integrin-mediated adhesion to FN substrates (Sechler and Schwarzbauer, 1998).

Fibronectin affects cell differentiation in many ways. Fibronectin has been shown to inhibit suspension-induced terminal differentiation of human keratinocytes via interaction with β1 integrins (Watt *et al*, 1993). In addition, FN inhibits insulin-induced adipocyte differentiation via promoting FN fibrillogenesis (Kamiya *et al*, 2002). However, FN is required for osteoblast differentiation via interaction with integrins, since interfering with interactions between FN and FN-binding integrins suppressed the formation of mineralized nodules and expression of genes characteristic of mature osteoblasts (Moursi *et al*, 1996; 1997).

Recent studies have shown that changes in FN matrix organization affect cell shape and function. For example, a mutant FN lacking the first seven Type III repeats ($FN\Delta III_{1-7}$) that contains all known cell binding sites but forms a fibril organization distinct from native FN, inhibited cell growth by specifically blocking G1 to S phase progression through reduced activation of a focal adhesion kinase (FAK) signaling pathway (Sechler and Schwarzbauer, 1998). These stimulatory (by native FN) and inhibitory (by $FN\Delta III_{1-7}$) effects of cell proliferation were reversed by addition of excess 70 kDa amino terminal fragment of FN that blocks matrix assembly. A fragment of FN corresponding to part of the first Type III repeat ($FNIII_{1-C}$) has been shown to reduce FN matrix from the cell surface disrupting the stress fibers and reorganizing the cytoskeleton into filopodia at the cell periphery through activation of Cdc42 (Bourdoulous *et al*, 1998). The $FNIII_{1-C}$ also inhibited proliferation of

adherent fibroblasts and endothelial cells by inactivating cyclin E-cdk2 kinase (Bourdoulous *et al*, 1998). These indicate that normal FN matrix assembly provides positive signals necessary for cell cycle progression.

1.9.4 Effects of Amino-terminal Fibronectin Fragments on Cell Functions

The binding of the amino-terminus of FN to cell surface receptors, termed matrix assembly sites, is mediated by the first five Type I repeats, which appear to function as a single-binding unit (Sottile *et al*, 1991). Fibronectin fragments and recombinant constructs missing the amino-terminal region are not assembled into a fibrillar matrix. In addition, the presence of excess amino-terminal FN fragments blocks the binding of intact FN to cell surfaces and inhibits matrix assembly (McKeown-Longo and Mosher, 1985).

The amino-terminal FN fragments (FN assembly domain) have been shown to co-localize with α5β1 integrins at sites of focal adhesions (Dzamba *et al*, 1994; Christopher *et al*, 1997). A recent study showed that adhesion of fibroblasts to immobilized amino-terminal fragments of FN induced the formation of focal adhesions distinct from those formed by the interaction of RGD sequence of FN and α5β1 integrin in terms of protein composition (Hocking *et al*, 1998). Cell adhesion to the amino-termimal fragments did not promote actin stress fiber formation and reduced levels of tyrosine phosphorylation of FAK and paxillin. It was also found that cytochalasin D upregulated β1-integrin-mediated cell adhesion to the amino-terminus of FN suggesting a role for actin distribution on the ligand-binding specificity of the α5β1 integrin (Hocking *et al*, 1998).

Soluble FN is commonly found in plasma and most body fluids. Fragments of FN can be produced by proteinase cleavage of the native FN. Fibronectin and FN fragments have been reported to be increased in inflammatory conditions such as in synovial fluid and plasma of osteoarthritic patients (Xie *et al*, 1992; Barilla and Carsons, 2000), in chronic wound fluid (Grinnell *et al*, 1992b) and in periodontal gingival crevicular fluid (Huynh *et al*, 2002). In contrast to native FN, the 29 kDa amino-terminal FN fragment (FN-N29-kDa) increased production of nitric oxide in cultured human chondrocytes (Gemba *et al*, 2002). The FN-N29-kDa has been

reported to activate FAK in human chondrocytes, but this was not mediated by phosphatidylinositol 3-kinase (PI-3K) nor α5β1 integrin (Gemba *et al*, 2002). A study on adherent endothelial cells (Christopher *et al*, 1999) showed that the 70 kDa amino-terminal FN fragment which contains the matrix assembly domain prevented cytochalasin D-induced reorganization of both FN matrix and actin stress fibers and also inhibited cell cycle progression. The addition of amino-terminal fragments of FN to cell monolayers also inhibited wound closure by 50%.

A *Staphylococcus aureus* FN receptor (FnBPA and FnBPB) exhibits a high affinity for the FN-N29-kDa, FN assembly domain. It is interesting to determine if *S. aureus* FnBPs affect FN assembly and cell functions.

1.10 Fibronectin Expression by Keratinocytes

Fibronectin is a critical early component of the clot and the forming granulation tissue. Initially, the blood clot contains plasma FN, which is later replaced by cellular FN produced by keratinocytes, fibroblasts and macrophages (reviewed in Häkkinen *et al*, 2000b). It is believed that migratory keratinocytes produce ECM molecules that they could use to support their own migration. It has been reported that human keratinocytes synthesize, secrete, and deposit FN *in vitro* (Kubo *et al*, 1984; Clark *et al*, 1985). Synthesis of FN by 7-day-cultured keratinocytes in low Ca^{2+}, serum free media has been reported to account for 2.9% of total synthesized protein, 26.5% of fluid phase protein secretion, and 4.3% of deposited ECM protein (Clark *et al*, 1985). Using immunostaining, FN was found in association with the surface of basal keratinocytes at the peripheral zone of the epithelial colonies, whereas suprabasal cells located in the center of the colonies were largely unstained (Nicholson and Watt, 1991). Distribution of FN on non-permeabilized keratinocytes has been described as a punctate or fibrillar pattern or diffuse patches, and as pericellular around each cell and the colony (Clark *et al*, 1985; Kubo *et al*, 1987; Nicholson and Watt, 1991). In permeabilized keratinocytes, FN was detected in a perinuclear pattern as an intracellular pool, and at the cell periphery along membrane processes (Larjava *et al*, 1990).

During wound repair, cells are induced to deposit alternatively spliced isoforms of cellular FN containing either the EDA or EDB regions. Studies in adult mice depleted of plasma FN have demonstrated that plasma FN is not required for normal skin wound healing (Sakai *et al*, 2001). It has been shown that EDA- but not EDB-containing FN is expressed by migrating keratinocytes in wounds and in culture (reviewed in Häkkinen *et al*, 2000b). FN isoforms containing the EDA domain have been shown to be more potent than those lacking the EDA domain in promoting cell adhesion, cell spreading and cell migration, irrespective of the presence or absence of the EDB segment (Hashimoto-Uoshima *et al*, 1997; Manabe *et al*, 1997). Furthermore, purified α5β1 bound more avidly to EDA-containing FN than EDA-negative FN (Manabe *et al*, 1997), suggesting that the effect of the EDA exon splicing is to improve binding affinity to integrins. The EDA region has also been shown to promote FN-induced cell cycle progression through up-regulation of integrin-mediated mitogenic signal transduction (Manabe *et al*, 1999). More recently, it has been shown that the EDA segment of FN is a ligand for integrins α9β1 and α4β1 (Liao *et al*, 2002).

1.11 Summary

Epithelium is a primary barrier between the external environment and internal tissues of the hosts. It provides a barrier to micro-organisms, toxins and various antigens. *S. aureus* interacts with host cells and may influence cell behaviour in many ways. *S. aureus* is the most common micro-organism in simple wound infection. It is commonly found in the nasal cavity and on skin. In the oral cavity, *S. aureus* can be found in about 24% and 64% of healthy adults and children respectively.

On tissue examination, *S. aureus* is found mainly in extracellular spaces. It can cause both localized infections (*e.g.* mucositis/stomatitis and soft tissue abscesses) and life-threatening systemic diseases (*e.g.* nosocomial septicemia, and infective endocarditis). Recently, *S. aureus* in the oral cavity has been considered to be an important source of infection in septic arthritis. How the oral mucosal resistance breaks down and develops mucositis; or the bacteria breach the epithelial barrier to cause systemic complications is not yet well-understood. In general, the

infection results from the ability of *S. aureus* to colonize on host surfaces and produce exotoxins and enzymes that destroy tissues and protect the bacteria from the host immune responses. Toxin-producing stains of *S. aureus* have been isolated from staphylococcal mucositis in some patients. Heavy colonization of the oral cavity with toxin-producing *S. aureus* may cause local mucosal damage. Therefore, bacterial growth supernatants from *S. aureus* which contain a variety of proteins, exotoxins and enzymes, are of interest for the study of microbio-epithelial interactions which may influence wound repair and keratinocyte behaviour.

There is increasing evidence that *S. aureus* can be found within non-professional phagocytes including keratinocytes. The ability of *S. aureus* to be internalized and survive within the cells is thought to be important for the bacteria to evade the host immune system and cause deep tissue or disseminated infection. The interaction between *S. aureus* and host cells has been proposed to occur through a bridging where FN is bound by *S. aureus* FnBPs as well as host cell $\alpha 5\beta 1$ integrin. It is interesting to see if this also applies for normal human keratinocytes. Recent *in vivo* studies have demonstrated the ability of a recombinant fragment of FnBPs (rFnBF) to prevent *S. aureus* abscess formation in a low-inoculum guinea pig model of wound infection (Menzies *et al*, 2002). Blocking bacterial entry to host cells may permit them to be more easily killed by extracellular defenses. *S. aureus* FnBPs exhibit a high affinity for the FN assembly site at the amino-terminus of the FN.

The presence of *S. aureus* FnBPs may have other effects on cell biology via FN. To date there have been very few studies addressing this topic. Recent studies using T lymphocytes have shown that, by binding to FN, immobilized recombinant fragments of FnBPs of *S. aureus* are able to provide a costimulatory signal resulting in T cell activation. Immobilized rFnBP can also mediate T lymphocyte adhesion under conditions of fluid shear stress.

1.12 Hypothesis

Staphylococcus aureus is an extracellular pathogen and heavy colonization with toxin-producing strains of *S. aureus* can cause mucosal damage. However, it is now clear that *S. aureus* is internalized by a variety of cells *in vitro* including keratinocytes. Studies in epithelial cell lines and endothelial cells have shown that the interaction between *S. aureus* and host cells occur through the *S. aureus* FnBPs and host cell integrin α5β1 with fibronectin bridge. It is hypothesized that *S. aureus* FnBPs and host cell integrin α5β1 play a key role in the interaction of *S. aureus* with primary keratinocytes and that the *S. aureus* FnBPs as well as its secreted products may affect keratinocyte functions.

1.13 Aims of the Study

The aims of the study were to investigate the effects of *S. aureus* on human keratinocytes. Specific investigations were:

1. To examine the factors influencing the adherence of *S. aureus* to keratinocytes.

2. To examine the role of FnBPs in the internalization of *S. aureus* by keratinocytes.

3. To examine the correlation between differences in intracellular levels of *S. aureus* and surface expression of the α5β1 integrin on keratinocytes.

4. To examine the role of host cell integrins α5β1 and αvβ6 in the internalization of *S. aureus* by keratinocytes.

5. To examine the effects of a recombinant D1-D4 repeat units of *S. aureus* FnBPB on the function of keratinocytes by investigating cell adhesion and migration, cell cycle progression and cellular fibronectin matrix polymerization.

6. To examine the effects of bacterial growth supernatants from three *S. aureus* strains (NCTC6571, 8325-4 and LS-1) on the function of keratinocytes by investigating cell morphology changes and proliferation, cell adhesion and migration.

7. To examine the effect of staphylococcal α-toxin on keratinocytes using α-toxin producing and knockout strains by investigating cell morphology changes and proliferation, cell adhesion and transmembrane flux of propidium iodide.

Chapter 2
Materials and Methods

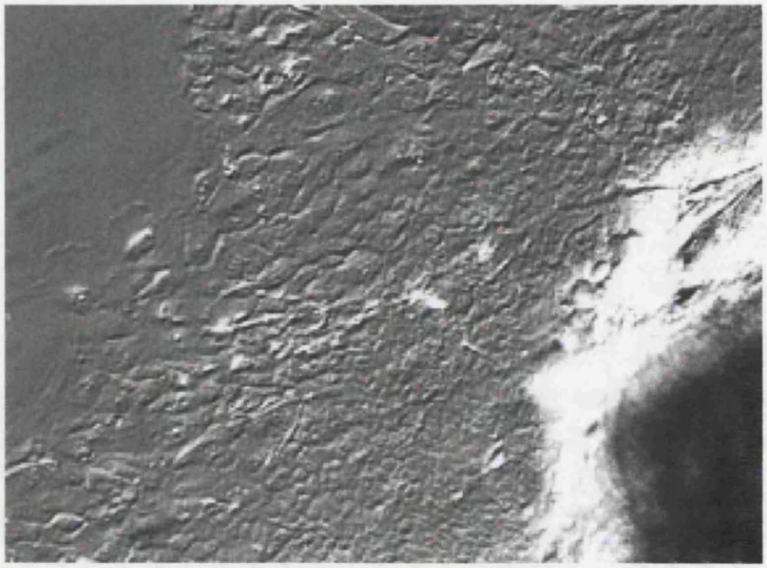
2.1 Introduction

Materials and methods described in this Chapter contain methods with general details that were used throughout the thesis. Specific applications or modifications of these methods and assays appear in the individual Chapters.

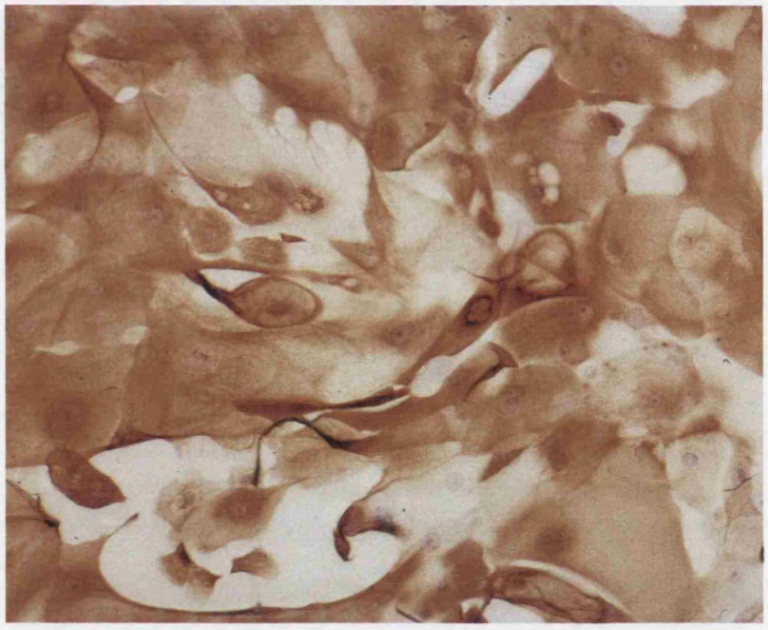
2.2 Epithelial Cells and Cell Culture Maintenance

The epithelial cells used in the study were HPV-transformed non-tumorigenic human epidermal keratinocyte cell line, UP (reviewed in Chapter 1, Section 1.5), and primary explant cultures of oral keratinocyte. The cells were maintained in keratinocyte growth medium (KGM). KGM was made up of three parts of Dulbecco's Modified Eagle's medium (DMEM; Gibco™, Invitrogen Ltd, Paisley, UK) plus one part of Nutrient Mixture Ham's F-12 (Ham's F-12; Gibco™) supplemented with 10% fetal calf serum (FCS; PAA Laboratories Ltd, Yeovil, UK), 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 10^{-10} M cholera toxin, 1.8×10^{-4} M adenine (Sigma®, Poole, UK), 100 IU/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml fungizone (Gibco™). To harvest cells for experiments, the cells were lifted from the culture plate by trypsinization with trypsin/EDTA (0.25%/0.05%; Gibco™) at 37°C for 10 to 15 min and collected by centrifugation at 1,000 rpm for 5 min at 4°C in the presence of KGM.

Normal human primary oral keratinocytes (NHK) were prepared as outgrowths from redundant normal gingival and mucosal tissues obtained with permission during minor oral surgical procedures at the Victor-Goldman Unit, Eastman Dental Hospital. Ethical approval was obtained. The oral tissues were kept in transport medium (see Appendix A) at 4°C and cultivated within 24 h as described by Freshney (1987) with some modifications. In brief, the tissue was briefly dipped in 70% alcohol and then rinsed in sterile phosphate-buffered saline (PBS) and KGM respectively. The tissue was then transferred to a sterile glass plate and unwanted connective tissue was dissected away. The remaining epithelium was finely chopped



(a)



(b)

Figure 2-1 Normal human oral keratinocytes (NHK) in culture.

(a) Phase contrast micrograph of NHK in an epithelial outgrowth from a primary explant after 8 days in culture. The outermost cells at the expanding edge exhibit areas where broad cytoplasmic processes extend out onto the culture substratum. Original magnification: x100.

(b) Immunocytochemistry of NHK from passage two culture shows expression of cytokeratins using polyclonal anti-cytokeratin MNF116. Original magnification: x250.

with crossed scalpels to about 1 mm cubes. Six pieces were transferred to each 25-cm^2 tissue culture flask (Nunclon™, Fisher Scientific UK, Loughborough, UK). KGM (6 ml per flask) was gently added and cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air without disturbing for one week. After one week, a substantial outgrowth of cells was observed (Figure 2-1a) and the growth medium was changed at this time and every 2-3 days afterward until approximately 18 days when each outgrowth was almost touching. The primary cultures of NHK were used mostly at passage two. The epithelial nature of the cells from the explant cultures was verified by positive staining with anti-cytokeratin antibody (MNF116; Dako Ltd, Ely, UK) which recognizes keratins 5, 6, 8, 17 and 19 (Figure 2-1b).

2.3 Determination the Number of Viable Cells using CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS assay)

The number of viable cells in proliferation, cytotoxicity and adhesion assays were determined using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega UK Ltd, Southampton, UK) according to the manufacturer's instructions. The CellTiter 96® AQ_{ueous} One Solution Reagent (CellTiter 96® reagent) contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS], and an electron coupling reagent (phenazine ethosulfate). The MTS tetrazolium compound in the assay acts as an artificial hydrogen-acceptor substrate for dehydrogenase activity in the metabolically active cells (Berridge and Tan, 1993). The MTS assay is based on the cellular reduction of the MTS tetrazolium compound by actively growing cells. The bioreduced MTS tetrazolium compound forms a colored formazan product which is soluble in tissue culture medium (Barltrop *et al*, 1991). This cytobiochemical assay can provide indirect reflection of cell number, in that dehydrogenase activity usually relates to cell number. It also reflects relative changes in cell metabolism induced by cellular toxins or stimulants.

To determine number of viable cells, 20 µl of the CellTiter 96® reagent was added into each well of 96-well plate. For the experiments where cell containing wells were washed before the MTS assay, fresh media or buffer (100 µl per well)

was added in each well and was allowed to equilibrate for 1 hour at 37°C before adding the CellTiter 96® reagent. The plate was incubated dark at 37°C for 1 hour. The absorbance was recorded at 492 nm using a microplate reader (model 340 ATTC, SLT Labinstruments, Salzburg, Austria). The absorbance values presented in the Figures or used for calculation the percentage of control and for statistical analysis were the values after substraction from the background absorbance of the CellTiter 96® reagent plus the media or buffer without cells. An example of effect of cell number on absorbance at 492 nm measured using the MTS assay is shown in Appendix A.

2.4 Epithelial Cell Proliferation Assay

Cell proliferation assays were performed in 96-well plates (Nunclon™). Subconfluent cells were harvested and resuspended in KGM. NHK or UP cells in suspensions were then plated in 96-well plates at a concentration of 10,000 cells per well. Keratinocytes were allowed to settle and attach for 24 hours producing subconfluent cultures before the experiments. The cell culture was washed twice and serum starved in DMEM for at least 2 hours. Triplicate wells were then incubated with each tested product. The number of viable cells in each well was quantified by the MTS assay as described previously.

2.5 Epithelial Cell Adhesion Assay

Cell adhesion assays were performed as described previously (Busk *et al*, 1992) with some modifications. Briefly, the bottom surface of each well of a 96-well plate was coated with 50 µl per well of 10 µg/ml human plasma FN (Sigma®) diluted in PBS at 37°C for 1 hour. The wells were washed twice with sterile PBS and then blocked with 0.5% bovine serum albumin (BSA; Sigma®) in PBS (BSA-PBS) at 37°C for 1 hour. After blocking, the wells were washed twice with sterile PBS and ready to use in the assay.

Subconfluent cells were harvested and washed once in serum free medium, SFM (3:1 mixture of DMEM:HAM's F-12). The cell suspensions in SFM were plated into each well at a concentration of 30,000 cells per well in the final volume of 100 µl/well. The cells were allowed to adhere for 2 hours at 37°C. Non-adherent cells

were removed by gently washing twice with PBS. SFM (100 µl per well) was added to each well and allowed to equilibrate for 1 hour at 37°C. The number of adherent cells was then quantified by the MTS assay as described in Section 2.3. The data were the absorbance values after substracting the background absorbance of the CellTiter 96® reagent plus SFM without cells.

2.6 Epithelial Cell Migration Assay

Modified Boyden chamber or transwell assays (haptotactic assay) were used to study cell migration. The haptotactic assay is a directional assay and measures the ability of a cell to sense and to respond to gradients in substrate concentration by coating only one (the lower) surface of the membrane with the substrate (Huttenlocher *et al*, 1996).

Haptotactic assays were performed using 6.5 mm Transwell®, polycarbonate membrane (8µm pore size, tissue culture treated) inserts in 24-well plates (Costar UK Ltd, Bucks, UK). The underside of each polycarbonate microporous membrane was coated with 10 µg/ml of human plasma FN (Sigma®) diluted in PBS (Figure 2-2). This was done by adding 250 µl of FN solution in each well of the 24-well plate and then incubating the inserts in the solution for 1 hour at 37°C. The inserts were then blocked with migration buffer, (3:1 mixture of DMEM:Ham's F-12 plus 0.5%BSA) before the assay for 1 hour at 37°C by adding 600 µl of migration buffer in the lower compartment of the Transwell®, then placing the insert containing 100 µl of migration buffer in its upper compartment into each well. At the same time, subconfluent cells were harvested and washed once in migration buffer. Migration buffer was removed from the upper chamber and replaced with 100,000 cells in 100 µl of migration buffer. Cells were allowed to adhere and migrate for 4 hours at 37°C.

The inserts were fixed in 10% formalin for 10 min and stained with 0.5% crystal violet in 10% ethanol for 10 min. The inserts were then dipped-rinsed in water and the cells on the upper surface were removed with a cotton bud. The membranes were air-dried overnight, excised from the insert and mounted on glass slides for microscopy. The migrated cells were counted at high power magnification (20x) from 6 randomly selected fields.

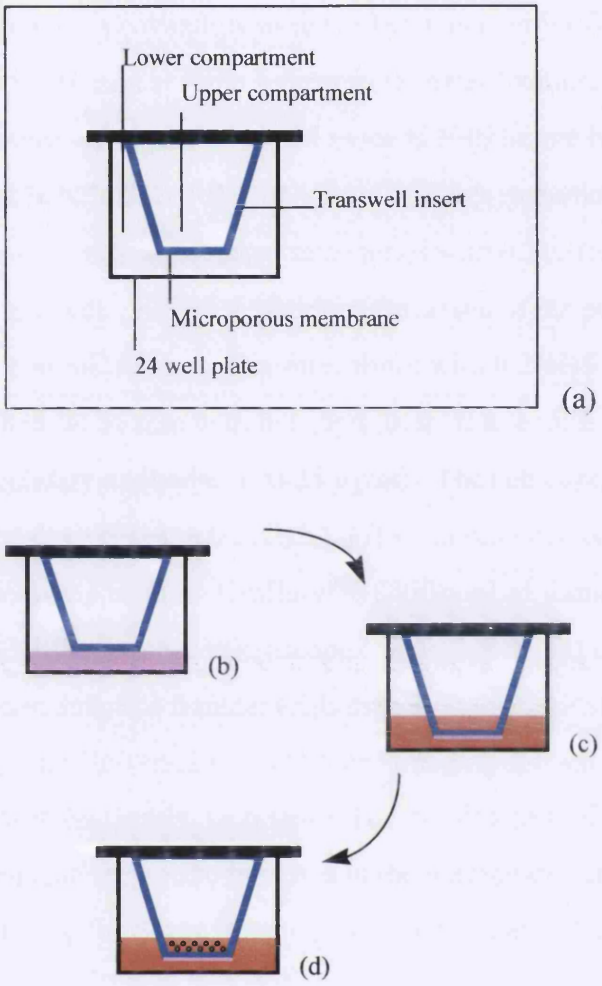


Figure 2-2 Haptotactic assay.

(a) Structure of the Transwell®.

(b) Coating the underside of the membrane with substrate.

(c) Blocking with migration buffer after coating.

(d) Replace the migration buffer in the upper compartment with 100,000 cells in 100 µl of migration buffer.

2.7 Indirect Immunocytochemistry

Adherent cells on glass coverslips were washed twice with PBS and fixed in 4% paraformaldehyde for 10 min at room temperature. After fixation, the cell containing glass coverslips were gently washed twice in PBS before blocking of non-specific binding sites using 2%BSA-PBS for 1 hour. Indirect immunofluorescence was performed as follows. Cells were rinsed three times with 0.2%BSA-PBS prior to incubation for 60 minutes with primary antibodies. Omission of the primary antibody served as the negative control. After rinsing three times with 0.2%BSA-PBS over 5 min, cells were incubated for 30 min with the appropriate fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (20-25 μg/ml). The cell containing coverslips were washed three times in 0.2%BSA-PBS and mounted on glass slides using glycerol-PBS mounting medium Citifluor™ (Citifluor Ltd, London, UK) and viewed with inverted Epifluorescence microscope (model Leica DM IRB) equipped with a Cohu monochrome interline transfer CCD camera (model 4900 series, Cohu, Inc., San Diego, USA), and Universal Leica Qfluoro Imaging software program (Leica Microsystems Wetzlar GmbH, Germany). The staining procedure was performed at room temperature. Antibodies used in the immunocytochemistry and immunofluorescent labeling for flow cytometry are listed in Table 2-1.

Table 2-1 Antibodies used for immunofluorescence stain.

Antibody to	*Clone*	*Dilution*	*Source*
Human fibronectin	Polyclonal	1:400 (1.25 μg/ml)	Sigma®
Human cellular fibronectin (EDA)	mAb IST-9	1:50 (10 μg/ml)	Oxford Biotechnology Ltd
α5 integrin	mAb P1D6	1:10	Dako Ltd
β1 integrin	mAb TDM29	1:10 (10 μg/ml)	Southern Biotech Assoc Inc
αvβ6 integrin	mAb E7P6	1:100 (10 μg/ml)	Chemicon Intl Ltd
αv integrin	mAb L230	15 μg/ml	Gift from JF Marshall
β6 integrin	mAb R6G9	1:100 (10 μg/ml)	Chemicon Intl Ltd

2.8 Immunofluorescent Labeling for Flow Cytometry

Cells were harvested and resuspended in ice-cold PBS containing 10% FCS (10%FCS-PBS) and transferred into flow cytometric tubes (Falcon®, Becton Dickinson, Cowley, UK) at approximately 500,000 cells per tube. The cells were washed once in ice-cold 10%FCS-PBS, then incubated with the primary antibodies for 1 hour on ice. The cells were then washed three times with ice-cold 10%FCS-PBS and incubated with the appropriate FITC-conjugated secondary antibody (20 μg/ml) for 30 min on ice. After washing three times, cells were resuspended in 500 μl of ice-cold 10%FCS-PBS ready for data acquisition and analysis using Becton-Dickinson FACScan and MacIntoch Power PC computer equipped with CellQuest research software (Becton-Dickinson). Fluorescence from 10,000 cells were measured per sample. Negative controls were labeled with secondary antibody alone. Propidium idodine (Sigma®), 10 μg/ml, was added for viability gating immediately prior to data acquisition. Mean fluorescence values were calculated by substracting the geometric mean fluorescence intensity (MFI, arbitrary unit) of secondary antibody labeled cells (negative FITC) from the MFI of primary antibody labeled cells.

2.9 *S. aureus* Strains and Growth Conditions

2.9.1 *S. aureus* Strains

All strains of *S. aureus* used in the experiments are listed in Table 2-2. Strains 8325-4 and its isogenic mutants, DU5883 and DU1090, were kindly supplied by Foster TJ, Department of Microbiology, Moyne Institute of Preventive Medicine, Trinity College Dublin, Ireland. Strains LS-1 and LSM were kindly supplied by Nair SP, the Division of Infection and Immunity, Eastman Dental Institute for Oral Health Care Sciences, University College London, UK.

S. aureus strain NCTC6571 is a laboratory strain commonly used as a control in the tube coagulase test and antibiotic sensitivity test. There are no isogenic mutants of this strain.

S. aureus strain 8325-4 is a laboratory strain commonly used in genetic studies. It is a derivative of NCTC8325 cured of the known three prophages (Novick, 1967). It has been passaged on laboratory media since before 1963 and still remains virulent in animal models of infection. It caused acute mastitis and death within 48 h for 60% of the mice inoculated (Bramley *et al*, 1989). However, virulent strains isolated today often are found to contain additional DNA sequences not represented in the standard strains. The additional DNA sequences are responsible for virulence properties, such as enterotoxin production, production of TSST-1 or resistance to methicillin (Iandolo *et al*, 1997). *S. aureus* strain 8325-4 does not produce any enterotoxins or TSST-1.

S. aureus strain 8325-4 possess two closely linked genes for fibronectin binding: *fnbA* and *fnbB* (Jönsson *et al*, 1991). Both genes are expressed and contribute to the ability of 8325-4 to adhere to solid-phase fibronectin (Greene *et al*, 1995). The production of FnBPA and FnBPB by 8325-4 occurs in roughly equal in amounts (Karlsson *et al*, 2001). An isogenic mutant (DU5883) disrupted in the *fnbA* (*fnbA*::Tc^R) and *fnbB* (*fnbB*::Em^R) genes of strain 8325-4 has been created by insertion mutagenesis (Greene *et al*, 1995). It was found that DU5883 was defective in adherence to fibronectin but not to fibrinogen (Flock *et al*, 1996).

Many toxins and enzymes are produced by *S. aureus* strain 8325-4. These include significant amounts of α-, β-, γ-, and δ-toxin or hemolysin, lipase, hyaluronate lyase, staphylokinase, metalloproteinase, proteases, nuclease and acid phosphatase. In contrast, production of coagulase and protein A is very low in most complex culture media. The strain does not produce any enterotoxins or TSST-1. Using stationary phase (8 h) culture supernatant and protein gel analysis, it was found that α-toxin (Hla; 33 kDa) was the most prominent protein in the 8325-4 secretion profile (Chan and Foster, 1998a).

Intracellular 8325-4 has been demonstrated in vacuoles and free within the cytoplasm of human umbilical vein endothelial cells and to induce endothelial cell apoptosis (Menzies and Kourteva, 1998). Strain DU1090 is a derivative of 8325-4 in which the *hla* (*hla*::Em^R) gene has been inactivated by allelic replacement (O'Reilly

et al, 1986). Comparisons of 8325-4 and its α-toxin-negative derivative DU1090 demonstrated that the capacity to produce α-toxin was associated with a greater propensity for apoptosis in endothelial cells (Menzies and Kourteva, 2000). It has been reported that *S. aureus* strain 8325-4 did not induce cytokine expression in human endothelial cells (Yao *et al*, 1995). Moreover, its growth medium possessed a product that inhibited cytokine gene expression in endothelial cells infected with another staphylococcal strain, Wb strain, by prevention of internalization of the bacteria by endothelial cells.

S. aureus strain LS-1 was isolated from a swollen joint of a spontaneously arthritic NZB/W mouse (Bremell *et al*, 1990; 1992). Its prototype is a TSST-1 producing strain. LSM is an isogenic mutant disrupted in the *fnbA* (*fnbA*::Tc^R) and *fnbB* (*fnbB*::Em^R) genes of strain LS-1. It was generated by co-transduction of the *fnbA*::Tc^R and *fnbB*::Em^R mutations from strain DU5883 into strain LS-1 (Ahmed *et al*, 2001). *S. aureus* strain LS-1 produces several exotoxins and enzymes particularly TSST-1 and enterotoxin A and B.

Table 2-2 Staphylococcal strains used in the study.

Strain	*Relevant characteristics*
NCTC6571	Wild-type, laboratory strain
8325-4	Wild-type, laboratory strain; derivative of strain NCTC8325 cured of known prophages; plasmid-free
DU5883	Mutant of 8325-4 defective in expression of FnBPA and FnBPB (*fnbA*::Tc^R, *fnbB*::Em^R)
DU1090	Mutant of 8325-4 defective in expression of α-hemolysin (*hla*::Em^R)
LS-1	Wild-type, pathogenic strain producing TSST-1, β-hemolysin and enterotoxins
LSM	Mutant of LS-1 defective in expression of FnBPA and FnBPB (*fnbA*::Tc^R, *fnbB*::Em^R)

2.9.2 Culture of *S. aureus*

The bacterial isolates were stored in Todd-Hewitt broth, THB (Oxoid Ltd, Basingstoke, UK) with glycerol (20% v/v) at -80°C. Purity of the bacterial stocks was checked by simultaneous subculture onto 5% blood agar (Oxoid) plates with visual inspection of colony morphology after overnight incubation at 37°C with aeration.

Frozen stock culture was streaked on a blood agar plate and incubated overnight at 37°C in air before selection of a single colony for overnight growth in 10 ml of freshly prepared THB in a 50 ml-plastic culture tube. All bacterial cultures were grown at 37°C in a shaking incubator. The mutant strains were grown in the presence of antibiotics to maintain their selective properties using 2 μg/ml of tetracycline (Sigma®) and 5 μg/ml (in culture broth) or 10 μg/ml (in blood agar) of erythromycin (Sigma®).

2.9.3 Preparation of Exponential Growth Phase *S. aureus*

To obtain exponential growth phase bacterial culture, a total of 40 μl of the overnight broth culture of *S. aureus* was transferred into fresh THB (10 ml in a 50-ml tube) and was allowed to grow about 3 hours for wild-type strains and 4 and half hours for mutant strains at 37°C in a shaking incubator. Bacterial numbers were estimated spectrophotometrically at a wavelength of 600 nm. The staphylococci were collected from broth culture by centrifugation at 3,000 rpm for 10 min at 4°C, washed twice in sterile PBS and suspended in serum-free and antibiotic-free defined medium, DMEM. Final staphylococcal concentrations were adjusted by reference to a standard curve relating bacterial count to optical density (see Appendix A-2). To minimize clumping, bacterial suspensions were mixed thoroughly at all steps of the preparation.

2.10 Bacterial Adhesion and Internalization Assays

Keratinocytes were plated at a concentration of 70,000 cells per well in 24-well culture plates (Nunclon™) and incubated at 37°C in 5% CO_2 for 2 days, until more than 90% confluent. Upon reaching confluency (approximately $1.7x10^5$ cells

per well for UP and $1.5x10^5$ cells per well for NHK), the cells were washed once with sterile PBS and incubated in DMEM. After 3 hours, the cells were rinsed twice with sterile PBS and blocked with 2%BSA-PBS for 1 hour. Exponential growth phase *S. aureus* suspended in DMEM were added to each well after removing of the blocking agent and rinsing twice with sterile PBS.

For microscopic examination, the cells were grown on 13-mm diameter glass coverslips in 24-well culture plates for 2 days and co-cultured with the bacteria as described above.

2.10.1 Bacterial Adhesion Assay

Following the incubation of keratinocyes with bacteria for indicated time, cell-containing wells were gently washed three times with sterile PBS to remove non-adherent bacteria. The keratinocytes were then detached from the plates and lysed by addition of 100 µl trypsin/EDTA (5 min at 37°C) and 900 µl of 0.1% TritonX-100 in PBS (2 min) respectively. Viable bacterial counts in colony forming unit (cfu) per ml were performed on blood agar plates using 10-fold dilutions (plate counting). The number of adherent *S. aureus* represented the actual adherent bacteria plus intracellular bacteria.

For microscopic examination, the cell-containing glass coverslips were dipped-rinsed three times in PBS to remove non-adherent staphylococci and fixed in 4% formaldehyde for 10 min before staining with Gram's stain technique and then mounting on glass slides with DPX mountant (BDH, VWR, London, UK). Adherent bacteria and keratinocytes were examined under a light microscope in which staphylococci stained dark blue on a pink background of cells.

2.10.2 Bacterial Internalization Assay

Internalization of *S. aureus* by keratinocytes was evaluated after gentamicin treatment. Briefly, at the end of the adherence assay, 1 ml of DMEM supplemented with 100 µg gentamicin was added to each well following the removal of non-adherent bacteria. The plates were incubated with the gentamicin for additional 2 hours at 37°C to kill extracellular bacteria. The plates were then washed twice with

PBS and the cells were trypsinized, lysed, and viable bacterial counts were performed as described above.

For microscopic examination, the internalized *S. aureus* were visualized after lysostaphin treatment. Lysostaphin hydrolyzes the peptidoglycan of all the staphylococci that synthesize peptidoglycan with pentaglycine cross-bridge (Navarre and Schneewind, 1999). Lysostaphin has been shown to remove *S. aureus* that is adherent to the endothelial cell monolayer but does not affect the viability of internalized bacteria (Vann and Proctor, 1987; Peacock *et al*, 1999). The bacterial adhesion assay was performed on glass coverslips using the method described above. At the end of the adherence assay, cell cultures were washed twice with PBS before adding lysostaphin (Sigma®) at 20 μg/ml in DMEM and incubating for 30 min at 37°C. The cell-containing glass coverslips were rinsed in PBS, fixed, Gram stained and mounted on glass slides as described previously.

2.11 Preparation of *S. aureus* Culture Supernatants

Overnight broth culture (800 μl) was transferred into 200 ml of fresh THB in a 500-ml Erlenmeyer flask. Staphylococci were allowed to grow in a shaking incubator at 37°C for 3 and half hours for the wild-type strains or 4 and half hours for mutant strains (exponential growth phase), and 18 or 24 hours (stationary growth phase) for both wild-type and mutant strains. Mutant strains of *S. aureus* were grown in the presence of antibiotics at all time. Bacterial culture supernatants were collected by centrifugation at 3,000 rpm at 4°C for 20 min and filtered through a 0.22 μm-pore size filter (Sarstedt Ltd, Beaumont Leys, UK). Aliquots of bacterial culture supernatants were stored at -80°C.

2.12 Assay for Hemolytic Activity in Bacterial Culture Supernatant

Alpha- or beta-hemolysin activity in bacterial culture supernatants was determined as described previously (Nilsson *et al*, 1999). The assays were performed in duplicate in 96-well microplates (round bottom). The hemolytic activity of each bacterial culture supernatant was assessed by titration against rabbit (alpha-hemolysin activity) or sheep (beta-hemolysin activity) red blood cells. Defibrinated sheep blood and rabbit blood were obtained from E & Q Laboratories Ltd,

Burnhouse, Bennybridge, UK. Erythrocytes were washed once and resuspended in PBS to a final concentration of 1% (vol/vol). Serial doubling dilutions of each *S. aureus* culture supernatant were performed in sterile PBS. The erythrocytes were added to the culture supernatants at equal proportions, and the mixture was incubated at 37°C for 30 min followed by incubation at 4°C for another 60 min. The highest dilution giving rise to hemolysis was defined as the hemolytic titer.

2.13 Expression and Purification of Recombinant FnBPBD1-D4 Protein

Escherichia coli strain M607 containing the recombinant plasmids of the protein encompassing the D1-D4 repeat region of *S. aureus* FnBPB (M607pREP4-pQE30-rfnbBD1-D4) was kindly provided by Nair SP, the Division of Infection and Immunity, Eastman Dental Institute for Oral Health Care Sciences, University College London. The recombinant protein encompassing the D1-D4 repeat region of FnBPB (rFnBPBD1-D4) contains an N-terminal extension of six histidine residues (6xHis-tagged protein). The theoretical pI and molecular weight are 4.56 and 17482.4 respectively as determined by the ProtParam Tool at http://ca.expasy.org.

2.13.1 Expression of rfnbBD1-D4

E. coli M607pREP4-pQE30-rfnbBD1-D4 were grown overnight in 4 x 20 ml Luria-Bertani broth (LB; Sigma®) containing antibiotics (100 μg/ml ampicilin, 25 μg/ml kanamycin, 20 μg/ml spectinomycin and 20 μg/ml streptomycin; all from Sigma®) at 30°C with shaking at 160 rpm. Four 1000-ml bottles, each containing 200 ml of LB, were inoculated with the non-induced overnight culture and incubated at 30°C with 160 rpm shaking for 2 hours. Expression of the protein was then induced by adding 200 μl of 1 M isopropylthio-β-D-galactoside (IPTG; BDH), final concentration approximately 0.9 mM. After 4 hours, the bacterial cells were harvested by centrifugation at 10,000 rpm for 20 min at 4°C. The cell pellets were stored overnight at -20°C before extraction and purification of the protein.

2.13.2 Purification of the rFnBPBD1-D4 Protein

The cell pellets were thawed on ice for 15 min before resuspending the cells in lysis buffer containing 8 ml of B-PER® Bacterial Protein Extraction Reagent (Pierce, Perbio Science UK Ltd, Tattenhall, UK) plus 20 mM imidazole, 80 µl of 120 mM phenylmethanesulphonyl fluoride (PMSF), and 8 µl each of 1 mM E-64, 10 mM leupeptin and 1 mM pepstatin A (all from Sigma®). The cells in lysis buffer were then incubated at 4°C for 1 h with mixing. The cell lysate was transferred to eppendorfs and cell debris was removed by centrifugation at 14,000 rpm at 4°C for 10 min. The clear supernatants were collected and pooled into a 15-ml falcon tube together with 1 ml of Ni-NTA agarose (Qiagen Ltd, Crawley, UK) and incubated with gentle mixing for 1 h at 4°C. The lysate-Ni-NTA mixture was then loaded into a polypropylene column of 1-ml capacity (Qiagen), and the column flow-through was collected for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The column was washed with 4 ml of B-PER® reagent and PBS, both containing 20 mM imidazole, followed by 4 ml of 2 mg/ml polymyxin B (Sigma®) in PBS to neutralise lipopolysaccharide activity. Following two further washes with 4 ml PBS, the bound protein was eluted with 4 x 0.5 ml PBS containing 250 mM imidazole.

The SDS-PAGE analysis of the flow-through, first two wash fractions and four eluates is shown in Figure 2-3. The SDS-PAGE was carried out using stacking and running gels with 5% and 12% acrylamide (see Appendix A), respectively. The proteins were separated under constant voltage set at 100 V for 1 hour or until the electrophoresis was complete. Separated proteins on the gel were stained overnight with Brilliant Blue G-colloidal Concentrate (Sigma®) and destained in water. The eluted fractions (lane E1 to E4) contained a single protein band with molecular weight of approximately 35 kDa. Only the first three eluted fractions were pooled and dialysed (membrane tubing with molecular weight cut-off of 3,500 Da; Spectrum®, Medical International Ltd, London, UK) against PBS at 4°C. The PBS was changed twice daily for 5 days. The protein concentration was determined by measuring the absorbance at 280 nm, using an absorbance of 0.220 for 1 mg/ml.

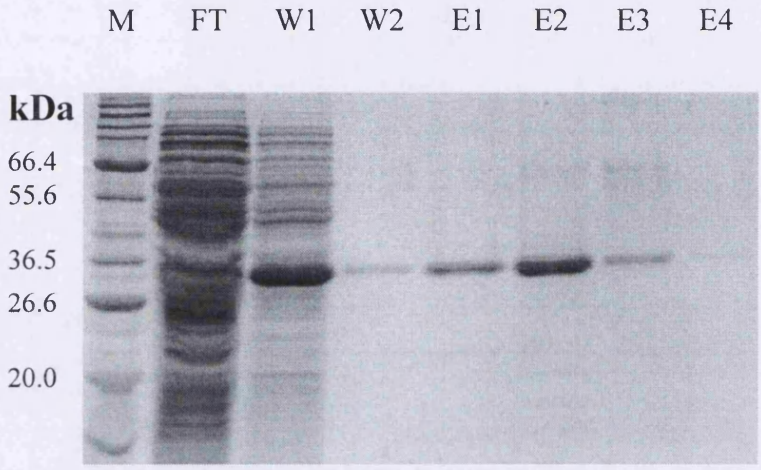


Figure 2-3 SDS-PAGE analysis of the fractions collected during the purification of rFnBPBD1-D4 protein using Ni-NTA agarose. Proteins were visualized by Coomassie staining. M, protein marker; FT, flow-through; W1, first wash with 20 mM imidazole in B-PER® reagent; W2, second wash with 20 mM imidazole in PBS; E, eluates in 250 mM imidazole in PBS. Each lane was loaded with equal volume of the protein fraction, except FT with half the volume.

2.14 Statistical Analysis

Data were analyzed using SPSS® package, version 11.0 (SPSS Inc, Chicago, IL, USA). The difference in mean values between two groups was evaluated using two independent samples t-test. When comparison the percentage value of the means with 100% of the control, statistical significance was performed using one sample t-test. The statistically significant level was set at the 0.05 level (two-tailed).

Chapter 3

Characteristics of UP Keratinocytes and NHK

3.1 Introduction

UP keratinocytes and primary cultures of human oral epithelial cells (NHK) were used extensively throughout the experiments described in this thesis. It is necessary to study the characteristics of the cells growing in the same culture condition as the experiments for bacterial adhesion and internalization assays.

The aim of the study was to examine the characteristics of UP keratinocytes and NHK in culture. These include cell morphology, localization of fibronectin and fibronectin-binding integrins, and levels of surface integrin expression.

3.2 Materials and Methods

3.2.1 Cell Culture

NHK from explant cultures and HPV-transformed UP keratinocytes were grown and maintained in KGM as described in Section 2.2. To prepare cells grown in 24-well plates for studies of cell morphology and indirect immunofluorescence, subconfluent cultures were harvested by trypsinization and cell suspensions in KGM were plated at a concentration of $7x10^4$ cells per well. The cells were allowed to grow for 2 days. To prepare cells for flow cytometry, cells maintained in 80-cm^2 flasks were grown to confluence before trypsinization.

3.2.2 Phase Contrast Microscopy

Cells grown to confluence in 24-well plate were observed under phase contrast microscopy.

3.2.3 Indirect Immunofluorescence

Indirect immunofluorescence was used to study the localization of FN and FN-binding integrins on human keratinocytes in culture. After two days in culture in 24-well plates, the cells were washed twice with PBS and serum-starved in DMEM for 3 hours before fixation with 4% paraformaldehyde. The cells were

immunofluorescently stained for human FN, integrin subunits α5 (P1D6), β1 (TDM29), αv (L230) and β6 (R6G9) or integrin αvβ6 (E7P6) as described in Section 2.7 without permeabilization. Dilutions and sources of the antibodies are listed in Table 2-1.

3.2.4 Flow Cytometry

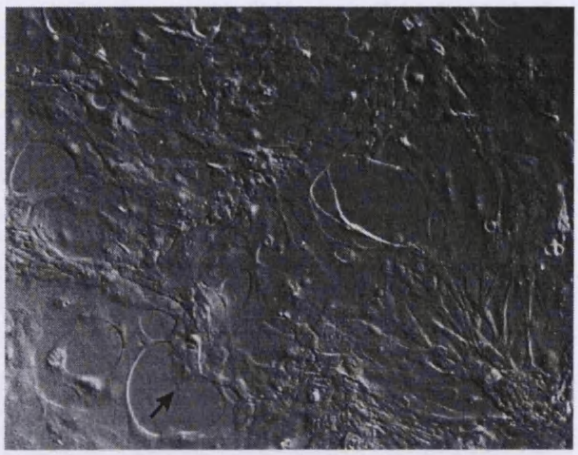
Surface expression of FN-binding integrins for both UP keratinocytes and NHK in cultures was determined by flow cytometry. Keratinocytes were harvested from confluent cultures. Cells were labeled for flow cytometry as described in Section 2.8 using antibodies for α5 (P1D6), β1 (TDM29), αv (L230) and β6 (R6G9) integrin subunits (Table 2-1). Mean fluorescence values were calculated by substracting the geometric mean fluorescence intensity (MFI, arbitrary unit) of secondary antibody labeled cells from the MFI of primary antibody labeled cells.

3.3 Results

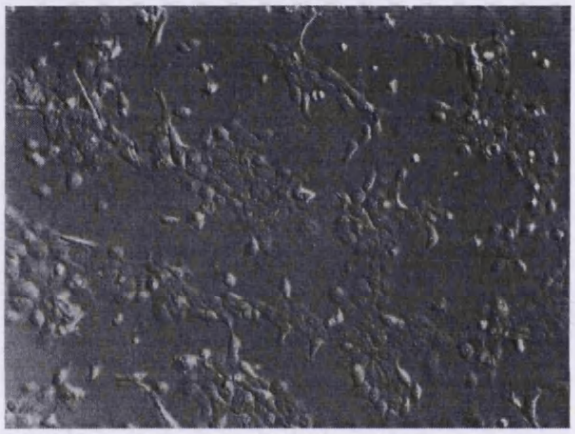
3.3.1 Morphological Studies

NHK and UP cells grown for 2 days in 24-well plates were more than 90% confluent. As shown in Figure 3-1a, NHK in culture viewed under phase contrast microscopy were generally flattened and epithelioid. The cells were heterogeneous in size and tightly adherent. They were similar or larger in size than UP cells. NHK in culture normally formed multilayers (stratified areas) interspersed with areas showing a single cell layer, particularly at the periphery of the epithelial sheets. The superficial cells were larger and squamoid. The cells at the expanding borders exhibited areas where broad cytoplasmic processes extended out onto the culture substratum (Figure 3-1a, arrow).

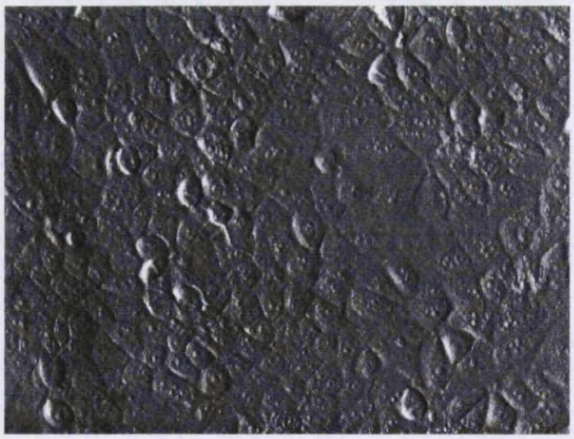
UP keratinocytes in culture viewed under phase contrast microscopy (Figure 3-1b and c) were generally similar to one another in size and shape. The cells were flattened and epithelioid. Stratified or multilayered areas were not observed in the confluent culture, however, occasional large vacuolated cells were noted.



(a)



(b)



(c)

Figure 3-1 Phase contrast micrographs of keratinocytes in culture. Original magnification: x100 for (a) and (b); x200 for (c).
(a) Confluent culture of NHK formed multilayers (stratification) interspersed with areas showing a single cell layer. The peripheral basal cells (arrow) showed broad expanding cytoplasmic processes. (b) Subconfluent culture of UP keratinocytes.
(c) Confluent UP culture showing dense epithelial cells without stratification.

3.3.2 Localization of FN on Cultured Keratinocytes (UP and NHK)

FN was generally detected at the pericellular areas, on the cell surfaces and on the glass coverslip. Figures 3-2 and 3-3 illustrate the localization of FN on UP and NHK respectively. On UP cell monolayers FN was strongly expressed around each cell. Deposition of FN in a granular or punctate pattern was also observed on the cell surfaces and the pericellular matrix on the glass surface. In stratified epithelial sheets of NHK, although the superficial cells were largely unstained, patches of granular deposition of FN could be seen in association with pericellular areas (Figure 3-3a), or granular distribution on the cell surfaces (Figure 3-3b) of some cells. In a single cell layer at the periphery of the keratinocyte colonies, granular deposition of FN was prominent at the cell-cell contact areas of the peripheral keratinocytes and in the pericellular matrix (Figure 3-3c).

3.3.3 Expression of FN-binding Integrins on UP Keratinocytes

Confluent cultures of UP cells consisted mainly of a single cell layer. FN-binding integrin subunits α5, β1, αv, and β6 were all detected in unpermeabilized UP cell monolayers. Integrin staining was heterogeneous, with some cells more intensely stained than others. In all cell populations, integrin staining showed a concentration at cell-cell borders.

The anti-α5 mAb P1D6 gave weak staining at the cell-cell borders (Figure 3-4a), whereas the anti-β1 mAb TDM29 gave intense staining (Figure 3-4b). The β1 integrins were absent from the free margins of the expanding cells at the periphery of epithelial sheets (Figure 3-4c).

The anti-αv mAb L230 predominantly stained cell-cell borders (Figure 3-4d), although immunoreactivity was detected on cell surfaces in a granular pattern. It was absent from the free margins of the peripheral keratinocytes. The αvβ6 integrin (mAbs E7P6 or R6G9) were detected occasionally as faint spotty staining at cell-cell borders and on cell surfaces, and as continuous strands along intercellular boundaries of some cells (data not shown).

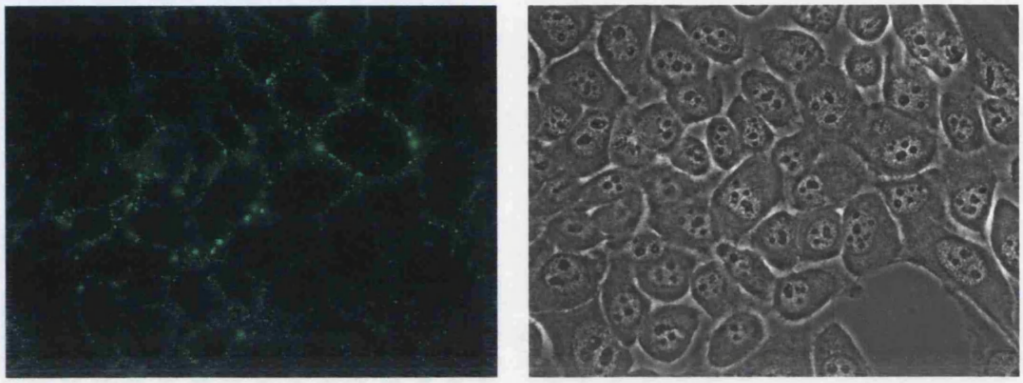


Figure 3-2 Localization of fibronectin on UP cell monolayers which had been grown for 2 days on glass coverslips in keratinocyte growth medium. Cells were stained with polyclonal antibody against human fibronectin without permeabilization. The corresponding phase-contrast micrographs are shown on the right. Note the presence of fibronectin in a pericellular distribution around each cell and on cell surfaces. Original magnification: x400.

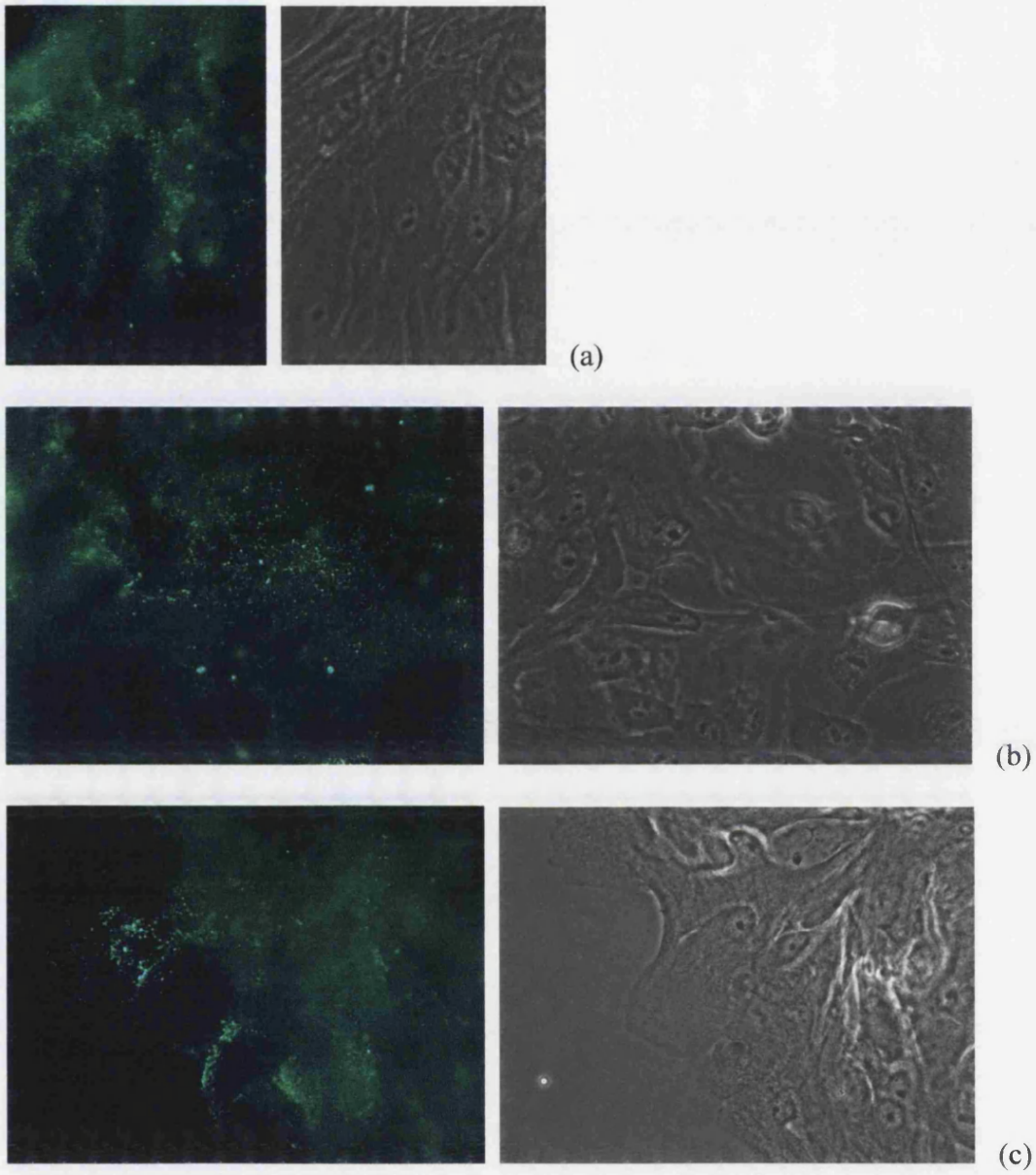
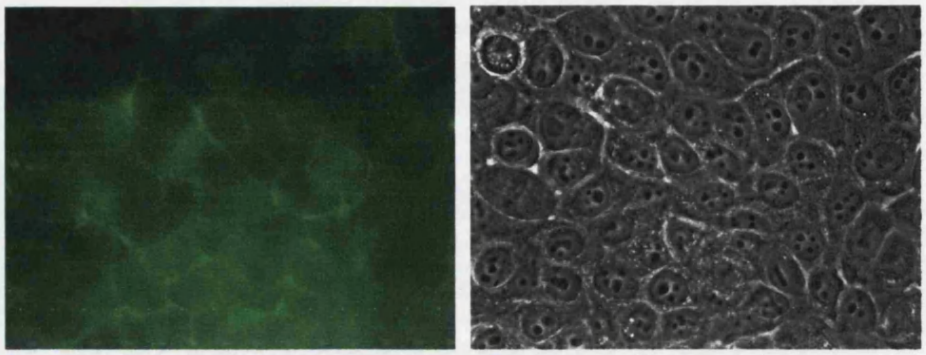
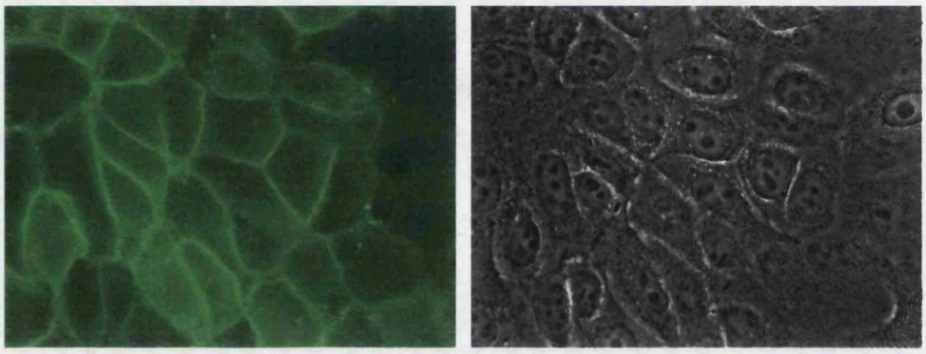


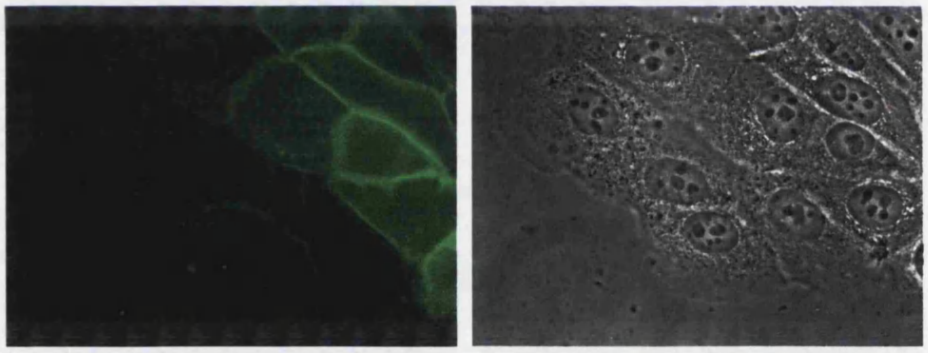
Figure 3-3 Localization of fibronectin on stratified epithelial sheets of primary oral keratinocytes (NHK) which had been grown for 2 days on glass coverslips in keratinocyte growth medium. Cells were stained with polyclonal antibody against human fibronectin without permeabilization. The corresponding phase-contrast micrographs are shown on the right. Note the presence of fibronectin at pericellular and cell surfaces of some superficial cells in the multilayered areas (a and b), at the cell-cell contact areas of the peripheral keratinocytes and in the pericellular matrix (c). The superficial cells in the multilayered areas were largely unstained. Original magnification: x400.



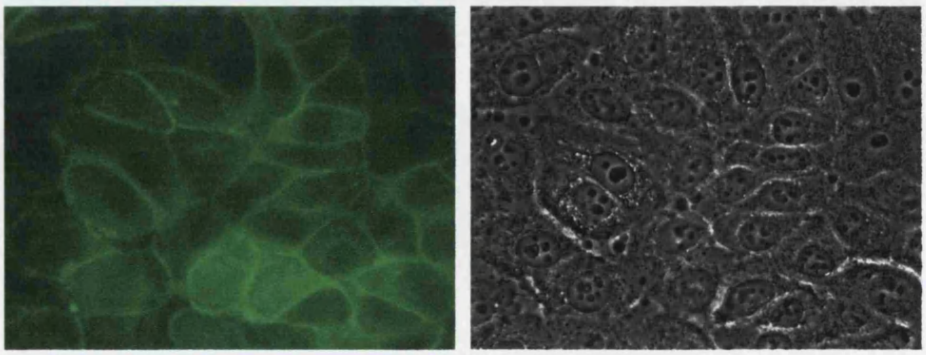
(a) The α5 subunit was detected occasionally at the cell-cell borders.



(b) Intense staining of the β1 subunit was detected at the cell-cell borders.



(c) The β1 subunit was absent from the free margins of the peripheral keratinocytes.



(d) The αv subunit was detected at the cell-cell borders.

Figure 3-4 Distribution of integrin subunits on UP cultures grown for 2 days on glass coverslips in keratinocyte growth medium. The corresponding phase-contrast micrographs are shown on the right. Original magnification: x630.

3.3.4 Expression of FN-binding Integrins on NHK

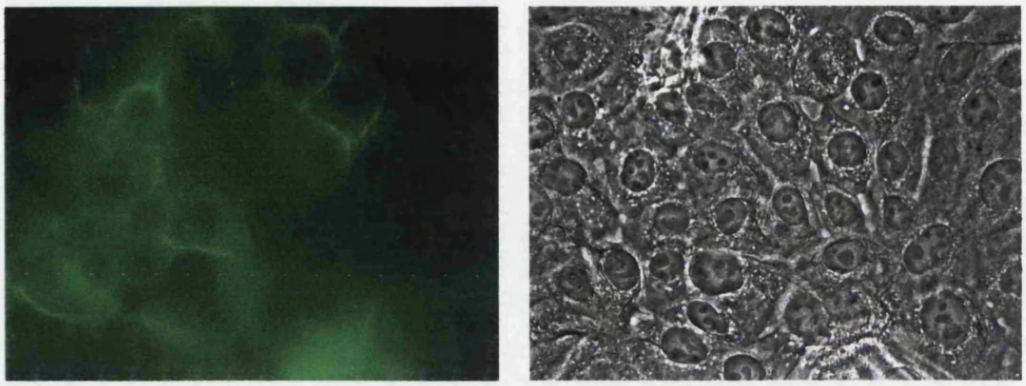
All of the integrin subunits were detected with expression mainly confined to the peripheral keratinocytes in a pericellular pattern with more intense staining along the cell borders. The keratinocytes that migrated upward and formed upper layers of the epithelial sheets showed weak or negative staining.

The α5 (Figure 3-5) and β1 (Figure 3-6) immunoreactivities were detected at the cell-cell contact areas of the peripheral keratinocytes. The α5 and β1 subunits were largely absent from the free margins of the peripheral cells, however, localized staining was sometimes seen.

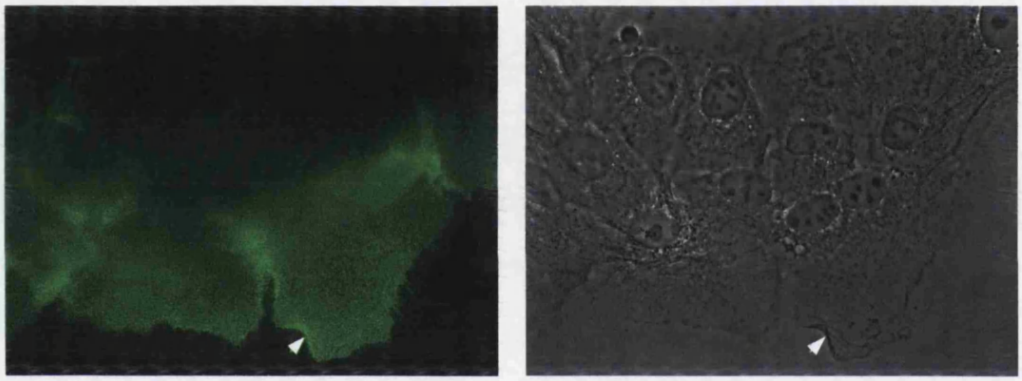
Intense staining of the αv subunit was detected at pericellular areas of the keratinocytes, but was largely absent from the free margins (Figure 3-7). Weak staining of the β6 subunit was detected occasionally at the cell-cell contact areas (Figure 3-8a). Localized staining at the free margins was also seen (Figure 3-8a, b). Intense staining around the nuclei and along the elongated intercellular processes (arrow) was observed in some expanding keratinocytes of the colonies (Figure 3-8c).Weak staining of the filopodia in NHK was also observed.

3.3.5 Surface Expression of FN-binding Integrins by Flow Cytometry

Surface expression, the mean fluorescence intensity, of integrin subunits α5, β1, αv and β6 on UP keratinocytes and NHK was shown in Figure 3-9. The integrin expression profile of UP keratinocytes was similar to that of NHK.

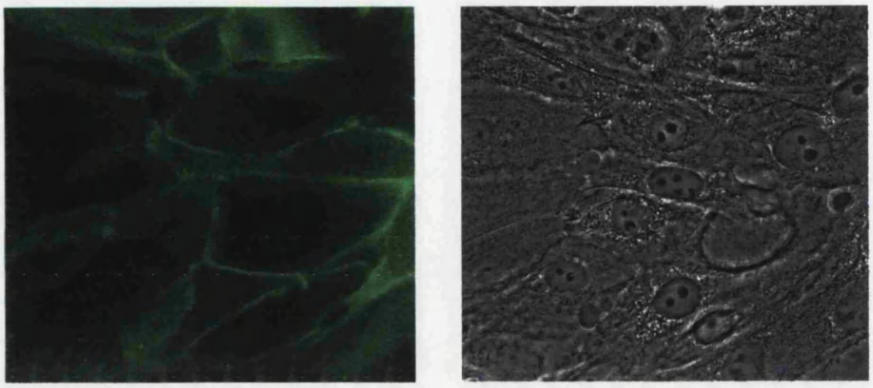


(a) The α5 immunoreactivity was detected occasionally at the cell-cell contact areas of the keratinocytes at a single cell layer.

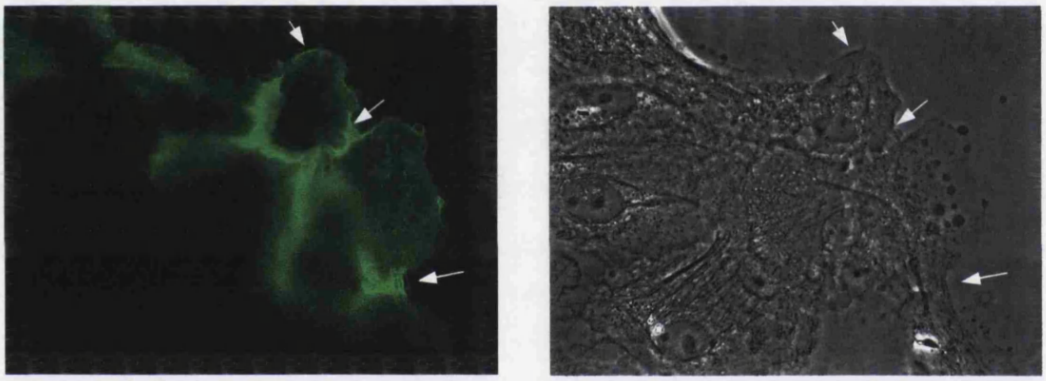


(b) The α5 subunit was largely absent from the free margins of the peripheral keratinocytes, but localized staining was sometimes seen (arrow).

Figure 3-5 Distribution of α5 integrin subunit on NHK cultures grown for 2 days on glass coverslips in keratinocyte growth medium. The corresponding phase-contrast micrographs are shown on the right. Original magnification: x630.



(a) Staining of the β1subunit was predominantly at cell-cell contact areas of the keratinocytes at a single cell layer.



(b) The β1 immunoreactivity was enriched on lateral surfaces (long arrow). It was largely absent from the free margins of the peripheral cells, but localized staining was sometimes seen (short arrow).

Figure 3-6 Distribution of β1 integrin subunit on NHK cultures grown for 2 days on glass coverslips in keratinocyte growth medium. The corresponding phase-contrast micrographs are shown on the right. Original magnification: x630.

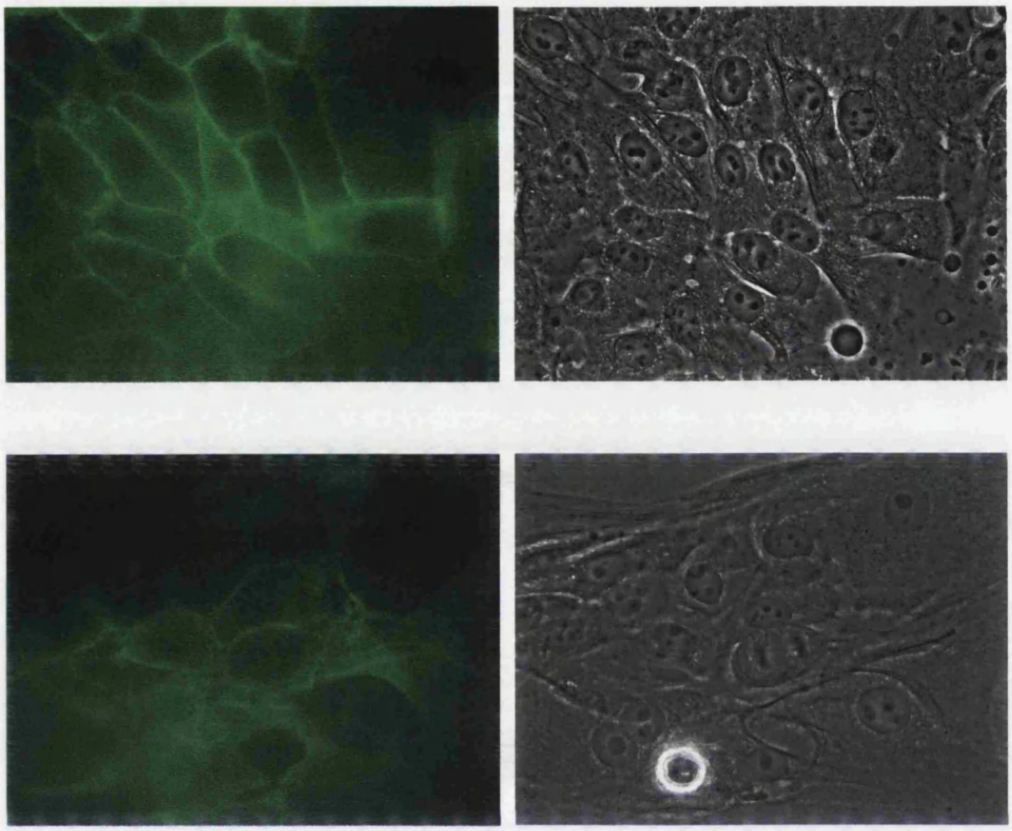


Figure 3-7 Distribution of αv integrin subunit on NHK cultures grown for 2 days on glass coverslips in keratinocyte growth medium. Intense staining of the αv subunit was detected at the cell-cell contact areas of the keratinocytes, but was absent from the free margins of the peripheral cells. The corresponding phase-contrast micrographs are shown on the right. Original magnification: x630.

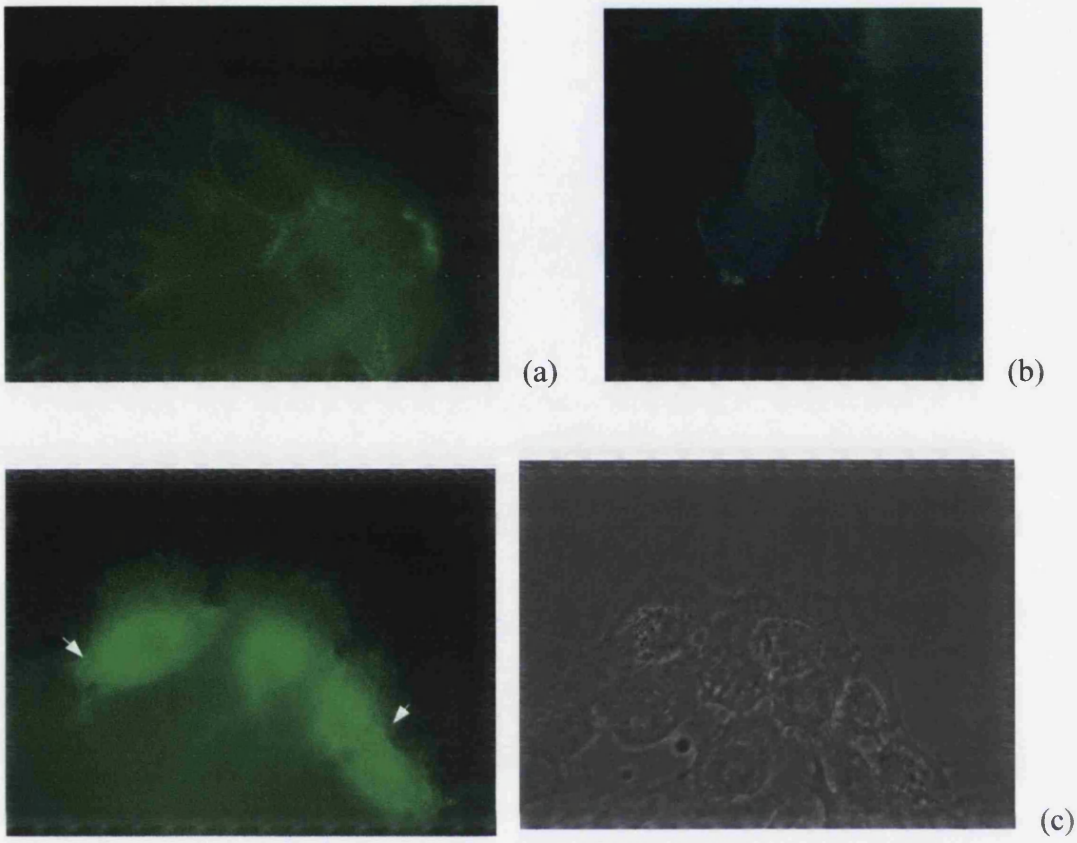


Figure 3-8 Distribution of β6 integrin subunit on NHK cultures grown for 2 days on glass coverslips in keratinocyte growth medium. The β6 immunoreactivity was detected occasionally at the cell-cell contact areas of the keratinocytes (a). Localized staining of the free margins was also seen (a-b). Intense staining around the nuclei and along the elongated intercellular processes (arrow) was observed in some expanding keratinocytes of the epithelial colonies (c). The corresponding phase-contrast micrograph is shown on the right. Original magnification: x630 for (a) and (c); x400 for (b).

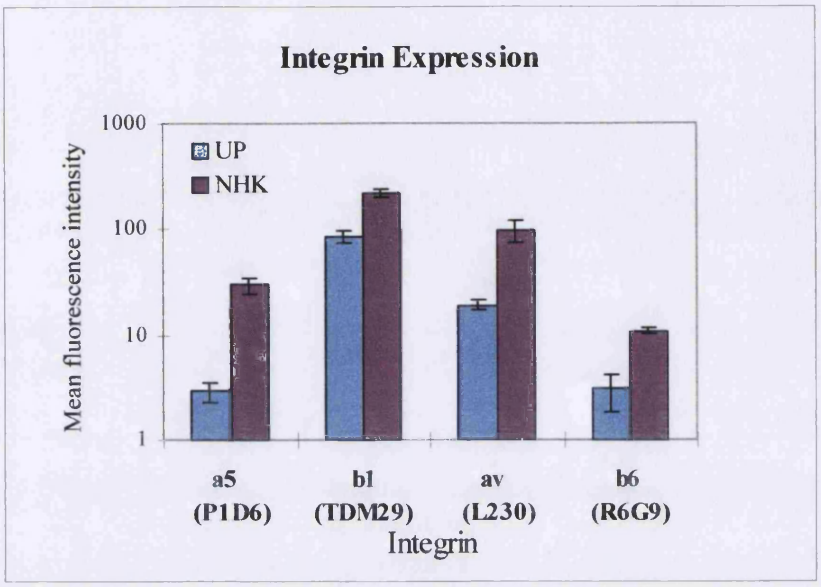


Figure 3-9 Geometric mean fluorescence intensity (arbitrary units, log scale) as measured by flow cytometry of UP keratinocytes and primary normal keratinocytes, NHK, labeled with anti-integrin antibodies. Negative control had secondary antibody only and was substracted from the results. Data are the mean ± standard deviation of three independent experiments.

3.4 Discussion

The results presented in this Chapter have demonstrated cell morphology and the distribution and localization of FN and FN-binding integrins, important host factors for *S. aureus* adhesion and internalization, of UP keratinocytes and NHK in culture. All the tested integrin subunits, α5, β1, αv and β6, were detected.

Fibronectin on non-permeabilized normal keratinocytes was found at the cell surface with some concentrated at cell-cell contact areas of the peripheral keratinocytes. Superficial cells located in the multilayered areas were largely unstained, in agreement with the previous observations of Nicholson and Watt (1991).

As reported previously (Adams and Watt, 1991; Marchisio *et al*, 1991), expression of the integrin subunits on normal keratinocytes was detected in areas consisting of a single cell layer at the periphery of epithelial sheets. The multilayered areas, which were covered by layers of terminally differentiating involucrin-positive cells, showed weak or negative staining. Localization of the α5 and β1 integrin subunits on NHK in this study is consistent with previous studies (Adams and Watt, 1991; Marchisio *et al*, 1991; Nicholson and Watt, 1991). The α5 and β1 subunits were concentrated at cell-cell boundaries and largely absent from the free margins of the peripheral keratinocytes.

The β6 subunit is absent in both normal human epidermis and normal oral mucosa (Breuss *et al*, 1995; Haapasalmi *et al*, 1996), although it is commonly expressed in squamous cell carcinomas derived from the oral mucosa (Jones *et al*, 1997). It has been shown that freshly isolated epidermal keratinocytes do not stain for αvβ6 integrin but begin to express this integrin after subculturing (Haapasalmi *et al*, 1996). In the present study, passage two cultures of NHK showed intense localized β6 immunoreactivity at the free edges of some peripheral keratinocytes. Occasional staining at the cell-cell contact areas was also observed.

As reported previously (Pei *et al*, 1991; 1992) and in the present study, UP keratinocytes grown to confluence in culture consisted of mainly a single cell layer. Integrin staining showed a concentration at cell-cell borders.

Flow cytometry showed that transformed UP keratinocytes had reduced surface levels of α5, β1, αv and β6 integrin subunits compared to NHK. This is consistent with the study by Hodivala *et al* (1994) comparing them with the parental normal epidermal keratinocytes.

In conclusion, UP keratinocytes and NHK expressed FN and integrins α5β1 and αvβ6 in culture condition used for the bacterial assays. UP cell monolayers contained mainly a single cell layer whereas NHK normally grew in multilayered epithelial sheets with some areas of a single cell layer, especially at the periphery.

Chapter 4

Adhesion and Internalization of *S. aureus* by Human Keratinocytes

4.1 Introduction

The oral mucosa represents the primary barrier between the oral environment and the deeper tissues. It also provides a barrier to micro-organisms, toxins and various antigens. Some orofacial infections such as angular cheilitis (MacFarlane and Helnarska, 1976; Ohman *et al*, 1986) and denture-induced stomatitis (Theilade and Budtz-Jorgensen, 1988) are usually complicated by the presence of *S. aureus*. *S. aureus* is also the most common pathogen in severe postoperative wound infections (fistulation and pus-formation) in patients undergoing major head and neck surgery involving direct wound communication between skin and mucosa of the oral cavity or the pharynx (Friberg and Lundberg, 1990). Adherence of *S. aureus* to tissue is an important process in colonization and subsequent establishment of infection. After colonizing the mucosal surface, secreted products from *S. aureus* can cause cell damage to human keratinocytes. Alpha-toxin producing strains of *S. aureus* can cause damage to human keratinocytes by forming small transmembrane pores (Walev *et al*, 1993). Other toxins, *e.g.* β-toxin and exfoliative toxin A, also cause damage to epithelial cells (Amagai *et al*, 2000; Kim *et al*, 2000).

Invasion of epithelial cells with intracellular persistence of micro-organisms is considered an important process for the bacteria to evade host defenses and antibiotics, and cause chronic relapsing infection (von Eiff *et al*, 2001), which may finally lead to deeper tissue or systemic infection. Although *S. aureus* is considered an extracellular pathogen, many *in vitro* studies have shown the existence of *S. aureus* within human nonprofessional phagocytes including keratinocytes (Nuzzo *et al*, 2000; Jung *et al*, 2001; Mempel *et al*, 2002), airway epithelial cells (Kahl *et al*, 2000), corneal epithelial cells (Jett and Gilmore, 2002), endothelial cells (Yao *et al*, 1995; Menzies and Kourteva, 1998) and osteoblasts (Jevon *et al*, 1999). *S. aureus* expresses an array of cell surface adhesins that bind to components of ECM. *S. aureus* FnBPs are likely the most important cell surface adhesins in the internalization of *S. aureus* into eukaryotic cells (reviewed in Section 1.8.1). The mechanism for *S. aureus* internalization into human endothelial cells (Massey *et al*,

2001), epithelial cells (Sinha *et al*, 1999) and osteoblasts is proposed to involve FN-dependent bridging between *S. aureus* FnBPs and host cell integrin α5β1. It is not known whether this mechanism is the case for all cell types. Interaction of *S. aureus* with keratinocytes may be important in gaining access to the deeper tissues and causing severe local and systemic complications.

Considering the significance of FnBPs in tissue invasion by *S. aureus*, the role of *S. aureus* FnBPs in adhesion and internalization of *S. aureus* with keratinocytes was investigated using isogenic mutant strains. The correlation between differences in intracellular levels of *S. aureus* and surface expression of the α5β1 integrin on keratinocytes was also investigated.

4.2 Materials and Methods

4.2.1 Keratinocytes

The keratinocytes used in this study were primary normal oral keratinocytes (NHK) and HPV-transformed epidermal keratinocyte line UP. A cell line derived from oral squamous cell carcinoma, H376 (Prime *et al*, 1990), was also used in some experiments. Surface expression levels of α5, β1 and αv integrin subunits of H376 cells are similar to those of normal oral keratinocytes (Sugiyama *et al*, 1993). All keratinocytes were maintained in KGM as described in Section 2.2.

4.2.2 *S. aureus* Strains and Culture Condition

S. aureus strains used in this study included NCTC6571, 8325-4, LS-1, DU5883 and LSM. Strains DU5883 and LSM are isogenic mutants, of 8325-4 and LS-1 respectively, defective in expression of FnBPA and FnBPB (*fnbA*::Tc^R, *fnbB*::Em^R). All *S. aureus* strains were grown in THB media. The mutant strains were grown in THB containing appropriate antibiotics to maintain their selected properties. Exponential phase cultures were used for all studies and prepared as described in Section 2.9.3. Final staphylococcal concentrations were adjusted by reference to a standard curve relating bacterial count to optical density (Appendix A-2). The actual number of the bacteria after adjusting (original inoculum) was determined by plate counting using 10-fold dilutions and was used to calculate the

percent of inoculum (100x[cfu of adherent or internalized bacteria/cfu of inoculated bacteria]).

4.2.3 Bacterial Adhesion and Internalization Assays

The bacterial adhesion and internalization assays were performed as described in Section 2.10. Suspensions of *S. aureus* ($5x10^7$ cfu) were co-incubated with the keratinocytes, in triplicate, for 90 minutes.

For microscopic examination, various concentrations of *S. aureus* were co-incubated with the keratinocytes grown on 13-mm glass coverslips. Non-adherent bacteria were washed off after indicated time. The adherent *S. aureus* were identified by Gram's stain as described in Section 2.10.1. The intracellular *S. aureus* were identified by Gram's stain after lysostaphin treatment as described in Section 2.10.2.

S. aureus strain NCTC6571 was used to examine the effect of bacterial vitality, dose and time of infection on bacterial adhesion to keratinocytes. The experiments were performed on glass coverslips and examined under a light microscope after Gram's stain. To kill bacteria, broth cultures of strain NCTC6571 were heated in a water bath at 65°C for 15 min before centrifugation to collect the bacteria. Bacterial death was confirmed by plating the samples on blood agar plates which showed no growth after 48 hours.

4.2.4 Flow Cytometric Analysis of α5β1 Integrin Expression on UP Cells Infected with *S. aureus* Strains 8325-4 and LS-1

UP cells grown to confluence for 2 days in 24-well plates were incubated with suspensions of *S. aureus* strain 8325-4 or LS-1 at a concentration of $5x10^7$ cfu per ml for 90 minutes, in quadruplicate, or incubated with DMEM alone. The plates were washed twice with PBS to remove non-adherent bacteria. The extracellular and adherent bacteria were killed by gentamicin treatment. After incubation for 1 hour in the presence of 100 μg/ml gentamicin, the plates were washed three times with PBS and the cells were harvested by trypsinization. The harvested UP cells from quadruplicate wells were pooled and immunofluorescently stained for the α5 (P1D6) or β1 (TDM29) integrin subunit (Table 2-1) as described in Section 2.8. Surface

expression of these FN-binding integrins was analyzed by flow cytometry with the use of propidium iodide (10 μg per ml) to gate out dead cells.

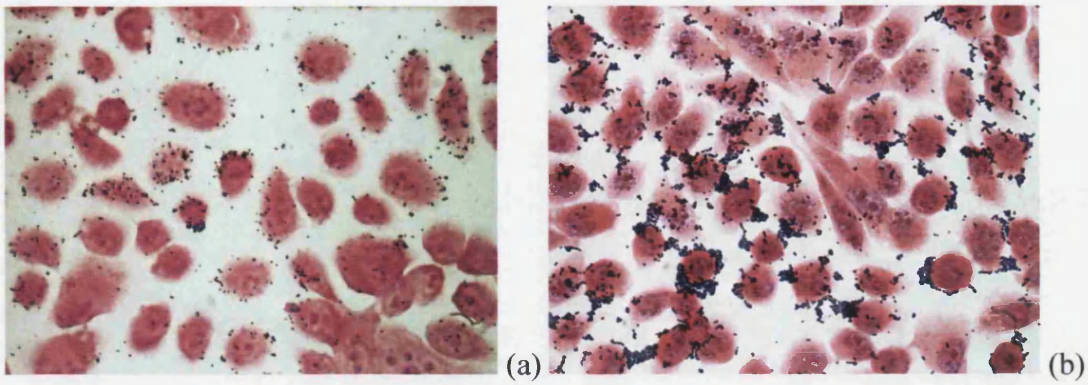
4.3 Results

4.3.1 Adherence of *S. aureus* to Human Keratinocytes

4.3.1.1 Effect of heat-killing, Bacterial Number and Co-incubation Time

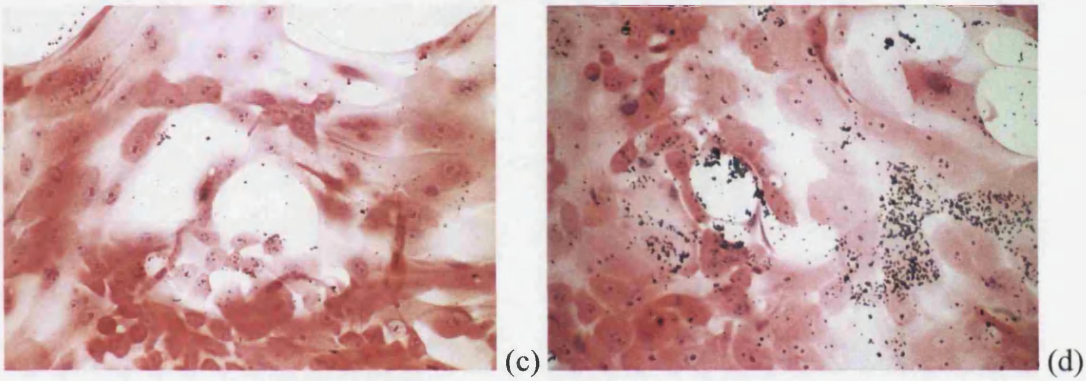
To measure the efficiency of *S. aureus* colonization on keratinocytes, the effect of bacterial vitality, bacterial number and incubation time on *S. aureus* adhesion was observed by light microscopy. When UP and NHK cultures were incubated for 90 min with live or heat-killed *S. aureus* strain NCTC6571 ($5x10^7$ cfu per well), the adhesion capacity of the heat-killed bacteria was less than that of the live bacteria (Figure 4-1). Thus, the later experiments were carried out with only live bacteria. It was observed that longer co-incubation time (90 versus 30 min) resulted in an increase adhesion of *S. aureus* to both UP and NHK (Figure 4-2). An increase in the inoculum size of *S. aureus* ($1x10^7$, $5x10^7$, and $1x10^8$ cfu per ml) also resulted in increased *S. aureus* adhesion capacity (Figure 4-3). However, large clusters of *S. aureus* varying in size were formed at the high concentration ($1x10^8$ cfu), particularly to UP cells. Accordingly, the lower bacterial inoculum at $5x10^7$ cfu (approximately 300 bacteria per keratinocyte) and 90 min co-incubation time were used throughout the following quantification experiments.

Heat-killed *S. aureus* Live *S. aureus*



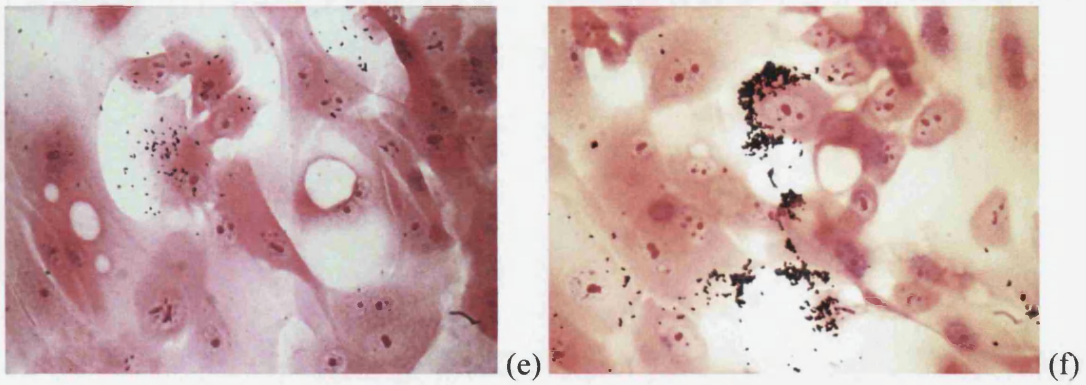
(a) (b)

UP (original magnification: x400)



(c) (d)

NHK (original magnification: x200)

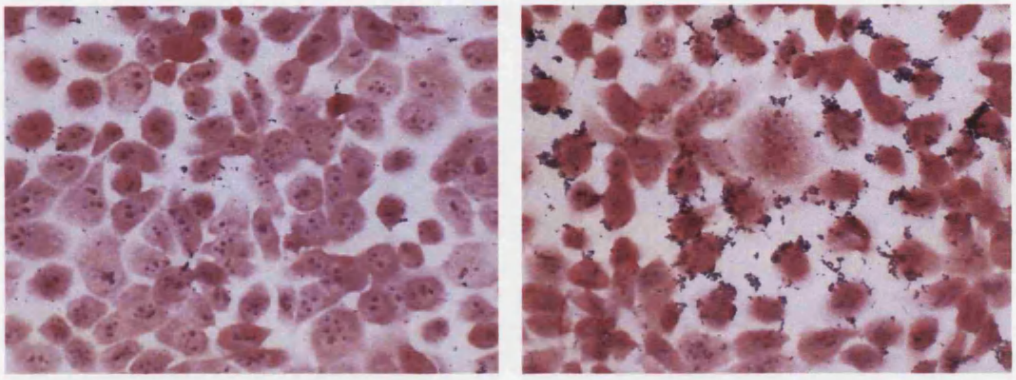


(e) (f)

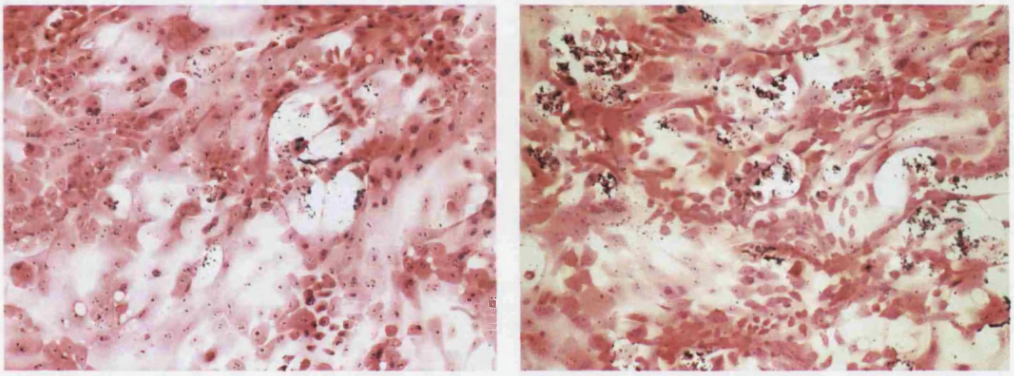
NHK (original magnification: x400)

Figure 4-1 Effect of bacterial vitality on the adherence of *S. aureus* to keratinocytes. Human keratinocyte, UP (a-b) or NHK (c-f), cultures grown for 2 days on glass coverslips in keratinocyte growth medium were incubated with either live (right) or heat-killed (left) $5x10^7$ cfu *S. aureus* strain NCTC6571 for 90 minutes. The nonadherent bacteria were washed off and the cells with attached bacteria were fixed with 4% formaldehyde and stained with Gram's stain.

30 minutes incubation 90 minutes incubation



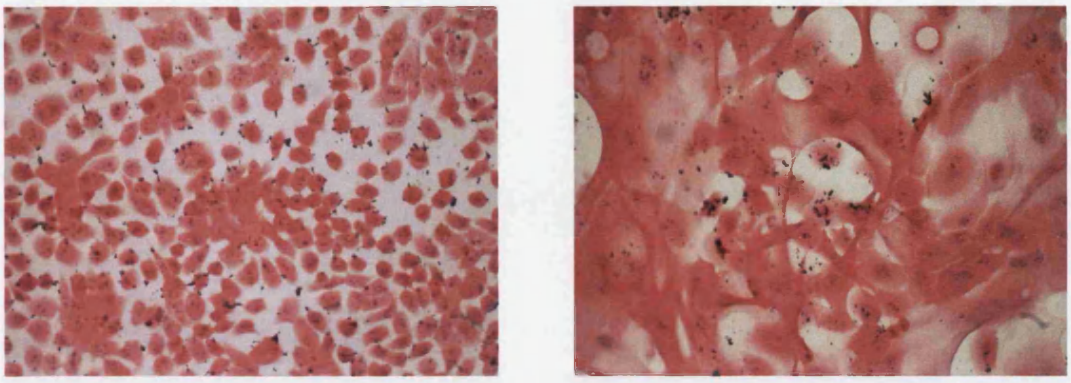
UP with *S. aureus* strain NCTC6571 (original magnification: x400)



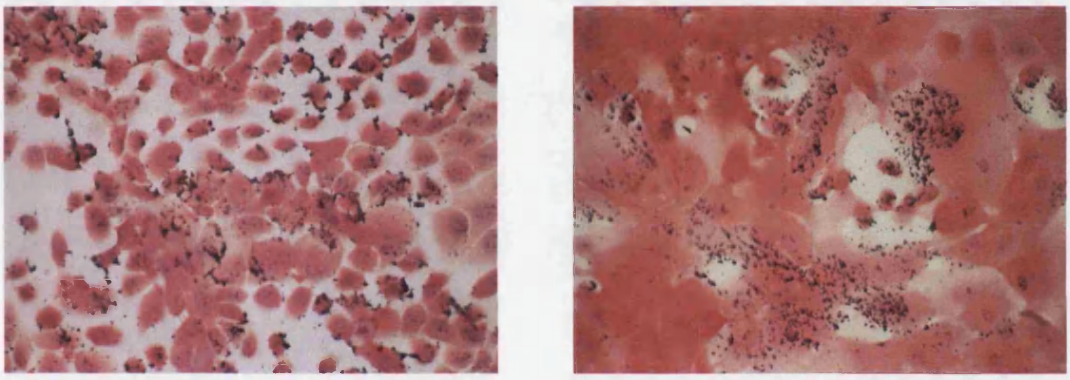
NHK with *S. aureus* strain NCTC6571 (original magnification: x100)

Figure 4-2 Effect of infection time on the adherence of *S. aureus* to keratinocytes. Human keratinocyte, UP (upper row) or NHK (lower row), cultures grown for 2 days on glass coverslips in keratinocyte growth medium were incubated with $5x10^7$ cfu *S. aureus* strain NCTC6571 for 30 (left) or 90 (right) minutes. The non-adherent bacteria were washed off and the cells with attached bacteria were fixed with 4% formaldehyde and stained with Gram's stain.

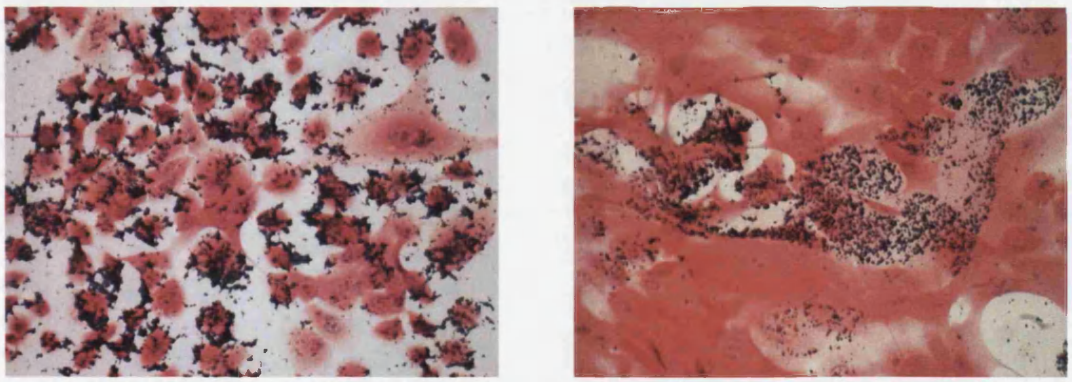
UP NHK



with $1x10^7$ cfu *S. aureus* strain NCTC6571 (original magnification: x200)



with $5x10^7$ cfu *S. aureus* strain NCTC6571 (original magnification: x200)



with $1x10^8$ cfu *S. aureus* strain NCTC6571 (original magnification: x200)

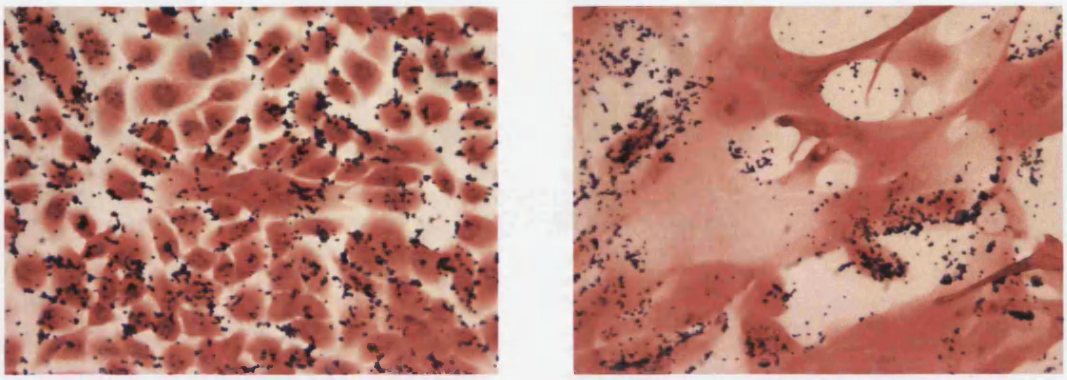
Figure 4-3 Effect of bacterial inoculum concentration on the adherence of *S. aureus* to keratinocytes. Human keratinocyte, UP (left) or NHK (right), cultures grown for 2 days on glass coverslips in keratinocyte growth medium were incubated with various concentrations of *S. aureus* strain NCTC6571 for 90 minutes. The non-adherent bacteria were washed off and the cells with attached bacteria were fixed with 4% formaldehyde and stained with Gram's stain.

4.3.1.2 Strain Dependent Adherence

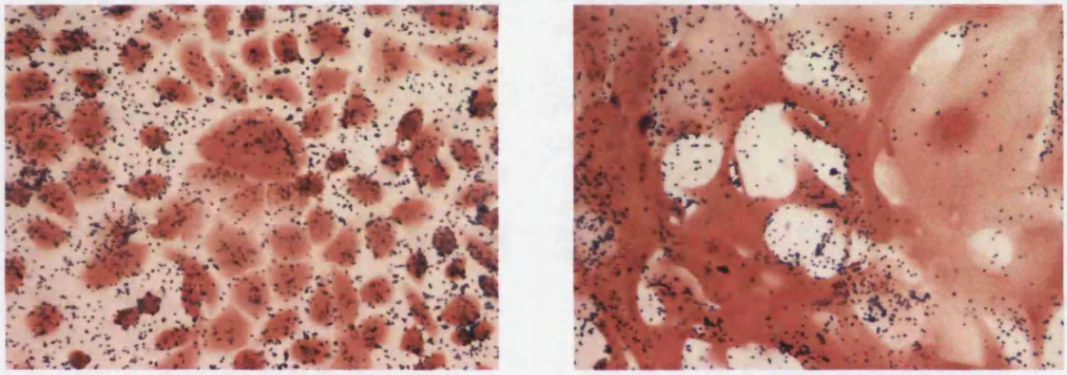
As shown in Figure 4-4, adhesion pattern of *S. aureus* varied from strain to strain. Adherence of strains NCTC6571 and LS-1 to UP cells was mostly at the pericellular region of each cell, similar to the FN localization on the cell monolayers shown in Figure 3-2. Small clusters of bacteria were observed. However, adherence of strain 8325-4 to UP cells was more diffused over the cell surfaces. For NHK, strain NCTC6571 adhered in clusters predominantly at the peripheral expanding keratinocytes (Figures 4-1f and 4-4a, right). Only a small proportion of the surface of the NHK showed bacterial adherence. Strains 8325-4 and LS-1, in contrast, diffusely adhered to epithelial sheets of NHK with rare clusters.

The ability of *S. aureus* to adhere to UP and NHK was also determined by plate counting of the number of adherent bacteria (Figure 4-5). The ability of strain 8325-4 to adhere to keratinocytes was higher than that of strain LS-1. The number of strain NCTC6571 adhered to NHK was higher than those of strains 8325-4 and LS-1.

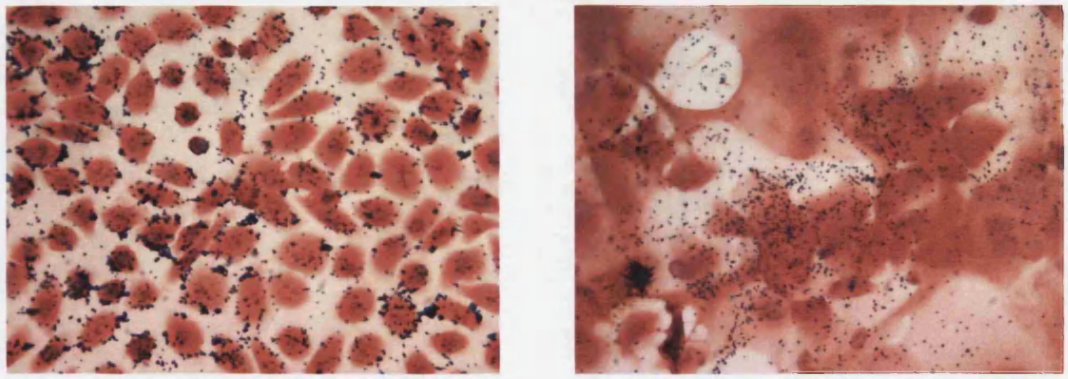
UP NHK



(a) with *S. aureus* strain NCTC6571 (original magnification: x400)

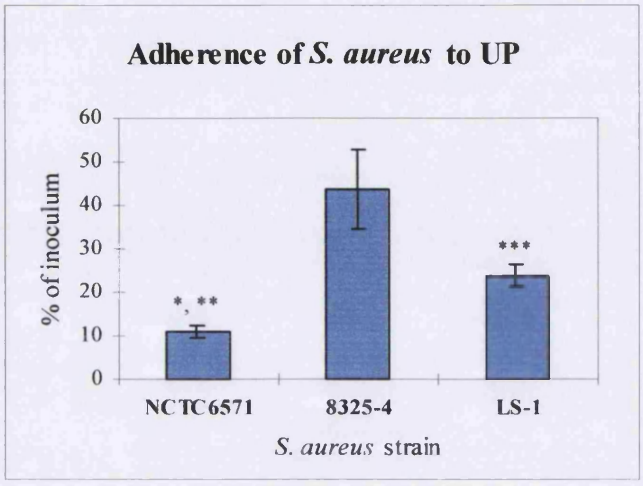


(b) with *S. aureus* strain 8325-4 (original magnification: x400)

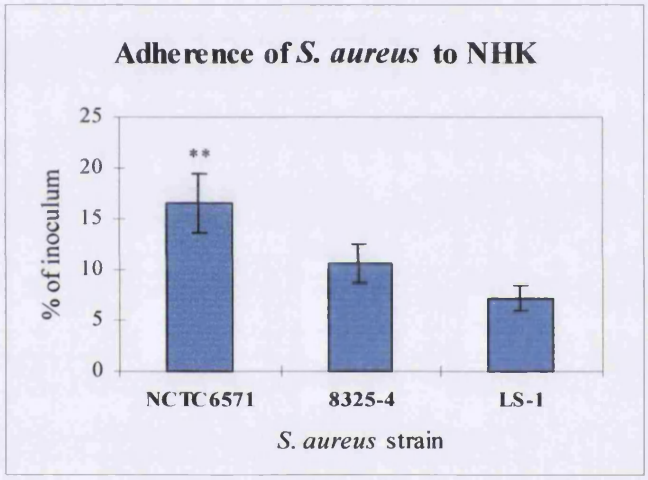


(c) with *S. aureus* strain LS-1 (original magnification: x400)

Figure 4-4 Adhesion pattern of *S. aureus* on keratinocytes. Human keratinocyte, UP (left) or NHK (right), cultures grown for 2 days on glass coverslips in keratinocyte growth medium were incubated with $5x10^7$ cfu *S. aureus* strain NCTC6571 (a), 8325-4 (b) or LS-1 (c) for 90 minutes. The non-adherent bacteria were washed off and the cells with attached bacteria were fixed with 4% formaldehyde and stained with Gram's stain.



(a)



(b)

Figure 4-5 The ability of *S. aureus* to adhere to UP (a) and NHK (b). The keratinocyte cultures grown for 2 days in keratinocyte growth medium were co-incubated with *S. aureus* strain NCTC6571, 8325-4 or LS-1 at a concentration of approximately $5x10^7$ cfu for 90 minutes. Non-adherent bacteria were washed off and the adherent bacteria (including internalized) were quantified by plate counting and presented as % of inoculum (100x[cfu of adherent bacteria/cfu of inoculated bacteria]). Data are the mean ± standard deviation from one representative experiment performed in triplicate.

*, significantly different ($p < 0.01$) from strain 8325-4;

**, significantly different ($p < 0.01$) from strain LS-1;

***, significantly different ($p < 0.05$) from strain 8325-4.

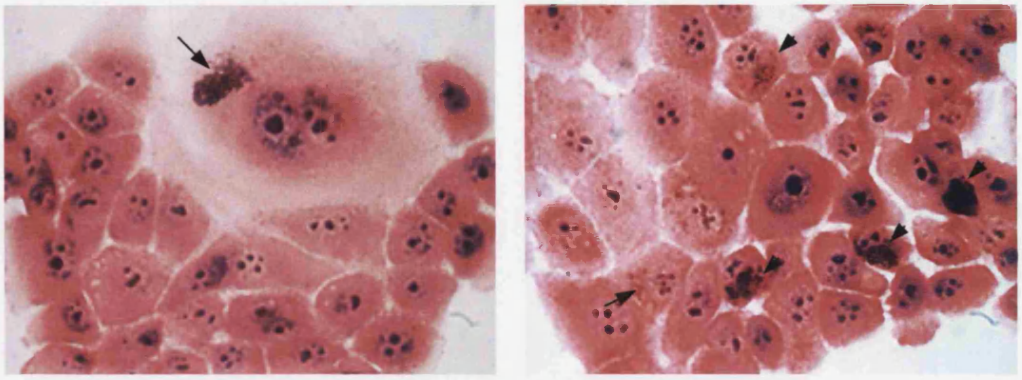
4.3.2 Internalization of *S. aureus* by Human Keratinocytes

4.3.2.1 Live and Heat-killed *S. aureus* can be Internalized by Keratinocytes

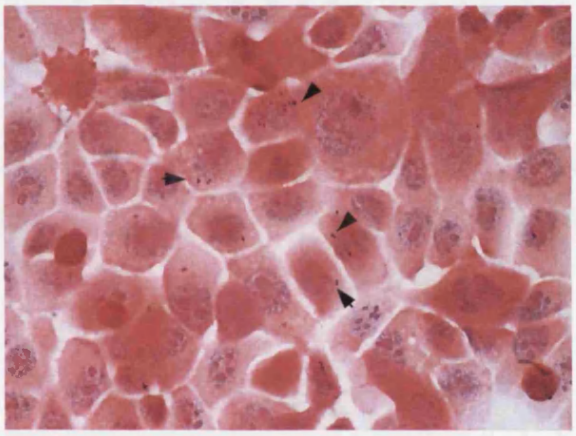
Photomicrographs demonstrating intracellular *S. aureus* are shown in Figure 4-6. Both live and heat-killed *S. aureus* could be found within the keratinocytes. However, not all keratinocytes in the culture contained intracellular bacteria. In addition, the number of intracellular bacteria within the keratinocytes was widely variable ranging from one or two bacteria to more than a hundred. In NHK, only a small proportion of the superficial cells contained bacteria. The presence of heat-killed *S. aureus* within the keratinocytes (Figure 4-6a) suggests that bacterial metabolic activity is not required for the internalization.

4.3.2.2 Internalization of *S. aureus* Strains by UP Keratinocytes and NHK

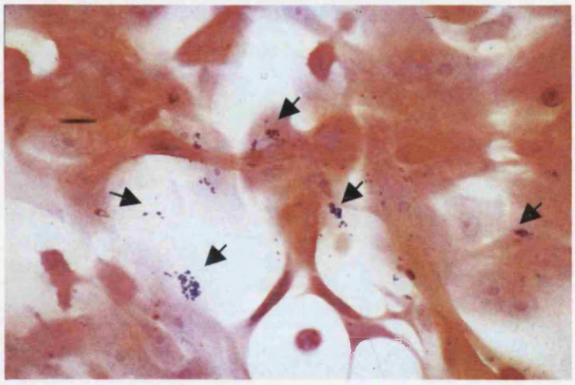
The capacity of three strains of *S. aureus* to become internalized by keratinocytes is shown in Figure 4-7. It was found that strain NCTC6571 was internalized by keratinocytes to a lesser degree than the other two strains tested, although it adhered to NHK with a higher degree. Strain LS-1 was internalized by NHK to a similar level as strain 8325-4. In contrast, LS-1 was highly internalized by UP cells, consistently showing 5- to 15-fold higher intracellular incorporation than strain 8325-4 (Figure 4-8b). In addition, this did not correlate with the levels of adhesion. The corresponding adhesion assays showed that strain LS-1 had a lower capacity to adhere to UP cells than strain 8325-4 (Figure 4-8a). This suggests that adherence of *S. aureus* to UP keratinocytes is not alone sufficient to mediate internalization.



(a) UP keratinocytes co-incubation with $5x10^7$ cfu heat-killed *S. aureus* strain NCTC6571 for 2 hours (left) or 6 hours (right). Original magnification: x400.



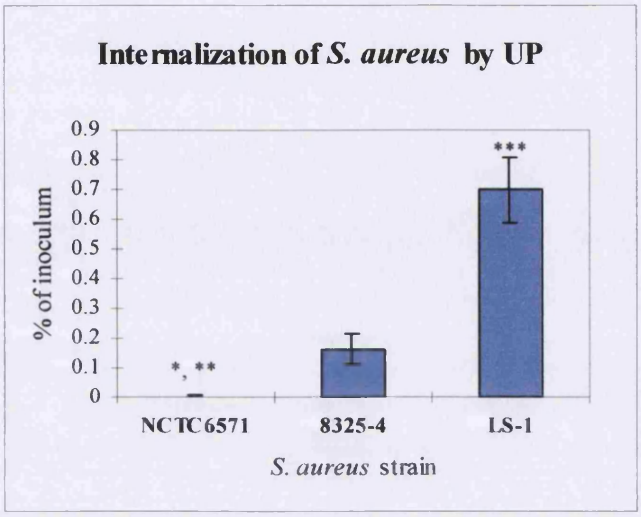
(b) Original magnification: x400.



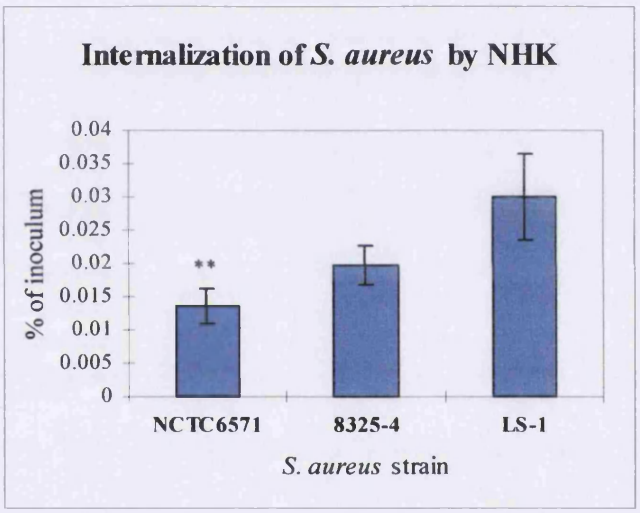
(c) Original magnification: x250.

UP keratinocytes (b) and NHK (c) co-incubation with $5x10^7$ cfu live *S. aureus* for 90 min.

Figure 4-6 Microscopic evaluation of the internalization of *S. aureus* by keratinocytes. Cultures on glass coverslips were washed and extracellular bacteria were lysed by the addition of 20 μg/ml lysostaphin for 20 min. Intracellular bacteria (arrow) were identified by Gram staining.



(a)



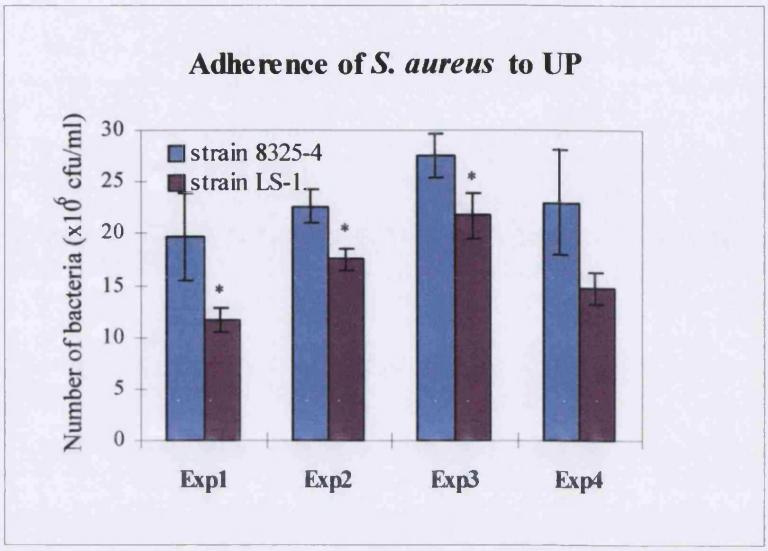
(b)

Figure 4-7 The capacity of three strains of *S. aureus* to be internalized by keratinocytes. UP (a) or NHK (b) were incubated with *S. aureus* strain NCTC6571, 8325-4 or LS-1 at a concentration of approximately $5x10^7$ cfu for 90 minutes. Internalized bacteria were quantified after gentamicin treatment for 2 hours by plate counting and presented as % of inoculum (100x[cfu of internalized bacteria/cfu of inoculated bacteria]). Data are the mean ± standard deviation of three replicate cultures.

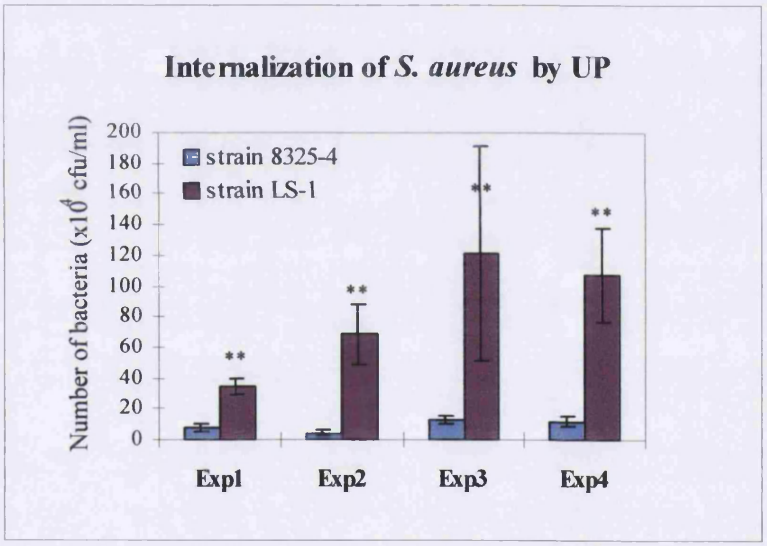
*, significantly different ($p < 0.01$) from strain 8325-4;

**, significantly different ($p \leq 0.01$) from strain LS-1;

***, significantly different ($p < 0.01$) from strain 8325-4.



(a)



(b)

Figure 4-8 Comparison of adherence (a) and internalization (b) of *S. aureus* strains 8325-4 and LS-1 by UP cells. The number of bacteria was quantified by plate counting. Data from each experiment are the mean ± standard deviation of three replicate cultures. *, $p < 0.05$; **, $p < 0.01$.

4.3.2.3 Surface Expression of α5 and β1 Integrin Subunits on UP Cells Infected with *S. aureus* Strains 8325-4 (low uptake) and LS-1 (high uptake)

The internalization of *S. aureus* by many eukaryotic cells has been shown to involve the host cell integrin α5β1 that binds to fibronectin. Surface expression levels of α5 and β1 integrin subunits on UP cells incorporated low (strain 8325-4) and high (strain LS-1) intracellular levels of *S. aureus* were analyzed by flow cytometry. The corresponding phase contrast micrographs showing the relation between the *S. aureus* and UP cells before harvesting were also taken. The phase contrast micrographs showed a relatively higher adherence capacity of strain 8325-4 to UP cell monolayer than strain LS-1 (Figure 4-9, top row). After the 90 min incubation of UP cells with *S. aureus* strains 8325-4 and LS-1, the expression of the α5 integrin subunit did not change. However, the expression of the β1 subunit was slightly decreased (Figure 4-9, bottom row). Although the reduction was small, it was consistent between the experiments. The reduction in the β1 integrin level of UP cells infected with strain LS-1 was not different from those infected with strain 8325-4. These data demonstrate that surface expression levels of the α5 and β1 integrin subunits on UP keratinocytes did not correlate with the levels of *S. aureus* internalization. The percentage of viable keratinocytes (propidium iodide excluded cells) after incubation with either strain 8325-4 (96.71 ± 0.93) or LS-1 (95.51 ± 1.15) was not significantly different from that of the control in DMEM alone (96.70 ± 1.58).

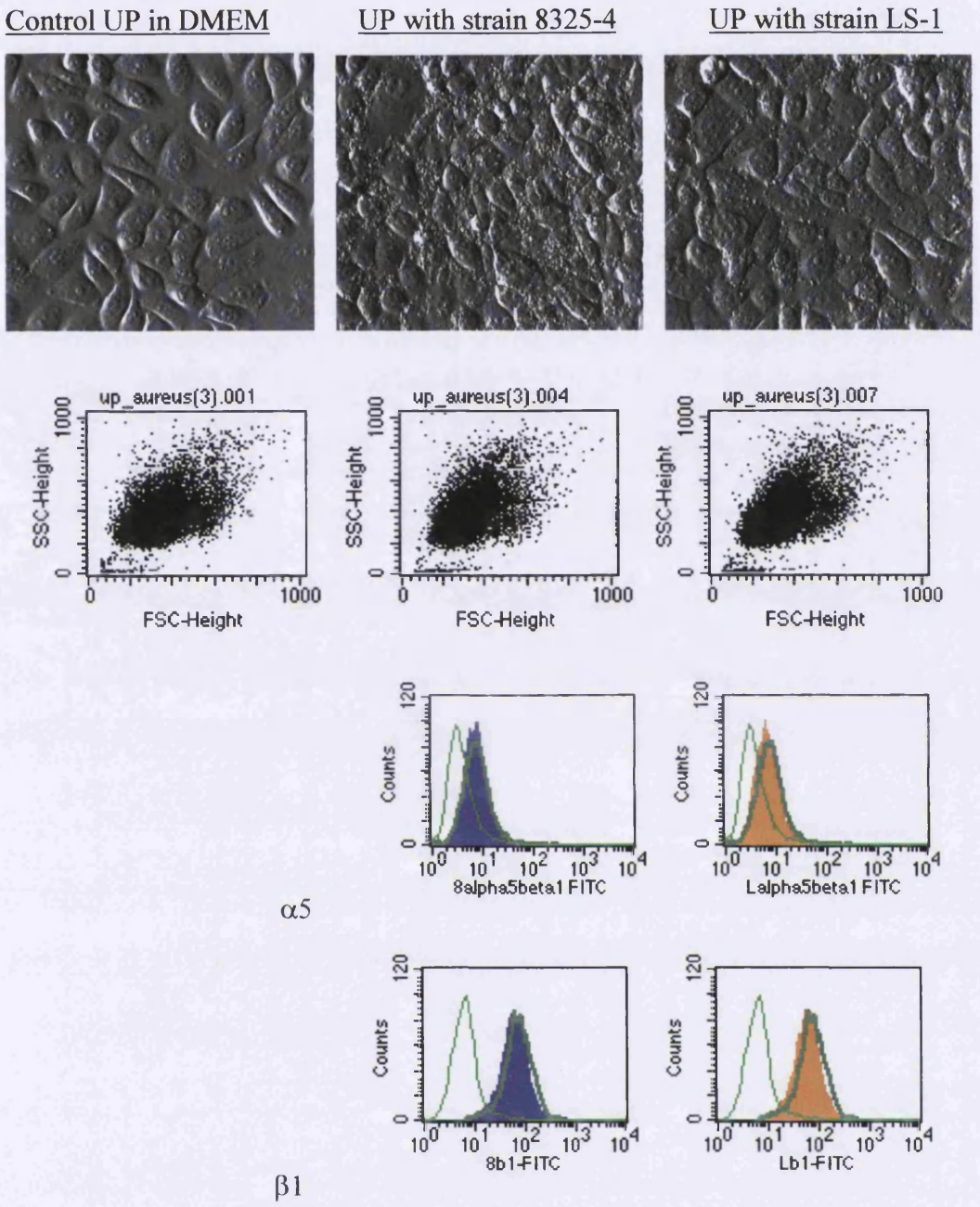
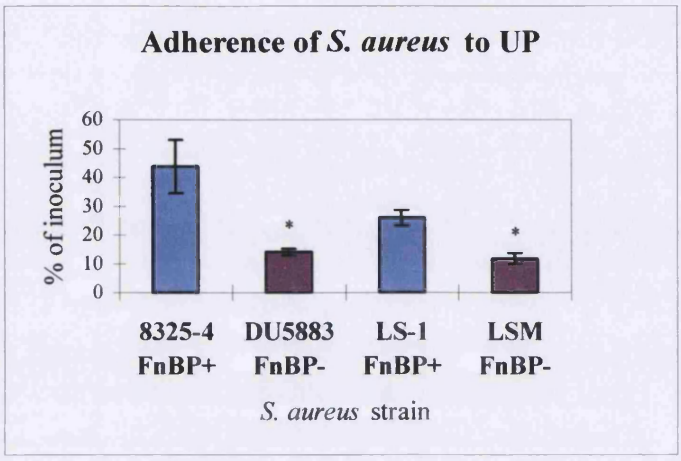


Figure 4-9 Evaluation of α5β1 integrin subunit surface expression (filled peak) on UP cells infected with $5x10^7$ cfu *S. aureus* strains 8325-4 (middle column) and LS-1 (right column) for 90 min. UP cells incubated in DMEM alone were used as positive control (thick line peak). The cells were harvested after 1 h gentamicin treatment, and immunolabeled with antibodies for α5 (P1D6) or β1 (TDM29) subunit. The negative control (thin line peak) had secondary antibody only. The corresponding phase-contrast micrographs were taken at 1 h after gentamicin treatment. The dot plots show forward versus side scatter distribution of control, 8325-4-infected, and LS-1-infected UP cells were similar. Data are from one of three independent experiments with similar results.

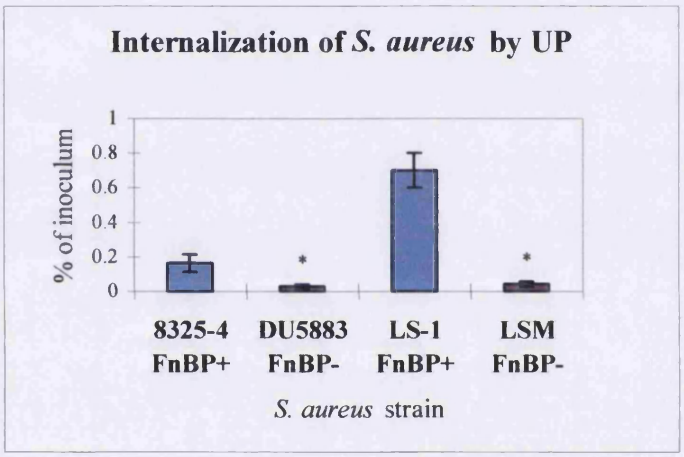
4.3.3 The Role of *S. aureus* Fibronectin-binding Proteins in Adherence of *S. aureus* to and Internalization by Keratinocytes

To determine the role of FnBPA and FnBPB in the ability of strains 8325-4 and LS-1 to bind to, and to be taken up by, UP and NHK, isogenic mutants disrupted in the genes for these proteins were compared to the parental strains. Evaluation of strain 8325-4 and its FnBP-deficient mutant DU5883 demonstrated a reduction of approximately 68% and 83% ($p \leq 0.01$) in bacterial adherence and internalization by UP cells respectively for the mutant DU5883 compared to the wild-type strain 8325-4 (Figure 4-10). The ability of the mutant strain LSM to adhere to UP cells was reduced by approximately 50% compared to the wild-type strain LS-1, and the internalization level was reduced by approximately 94% ($p \leq 0.01$; Figure 4-10). These results demonstrate the important role of the FnBPs in the adhesion and internalization process of *S. aureus* with UP keratinocytes.

In contrast to immortalized UP keratinocytes, the reduction in adherence (Figure 4-11) and internalization (Figure 4-12) of the 8325-4 FnBP-deficient mutant (DU5883) to primary normal keratinocyte NHK in culture was not observed in all three sources of the NHK tested (NHK12, 15, 22). The reduction in adherence and internalization of the LS-1 FnBP-deficient mutant (LSM) to NHK12 was approximately 50% and 60% respectively ($p = 0.01$), but this was not consistent between experiments with other sources of NHK. The apparent decrease in internalization of the mutant strain LSM by NHK12 corresponded to the reduction in adhesion to the cells. In NHK15, however, adherence and internalization of the mutant strain DU5883 were significantly increased.



(a)



(b)

Figure 4-10 The role of *S. aureus* fibronectin-binding protein (FnBP) in adherence (a) and internalization (b) of *S. aureus* by immortalized UP keratinocytes. The UP cells were grown for 2 days in keratinocyte growth medium and incubated with *S. aureus* strains 8325-4 and LS-1 and their respective isogenic mutants DU5883 and LSM (FnBP-) at a concentration of approximately $5x10^7$ cfu for 90 minutes. Non-adherent bacteria were washed off and the adherent bacteria (including internalized) were quantified. The internalized bacteria were quantified after 2 hours of gentamicin treatment. Data are presented as % of inoculum (100x[cfu of adherence or internalized bacteria/cfu of inoculated bacteria]). Data are the mean ± standard deviation from one of three experiments performed in triplicate with similar results. *, significantly different ($p \leq 0.01$) from the parental strain.

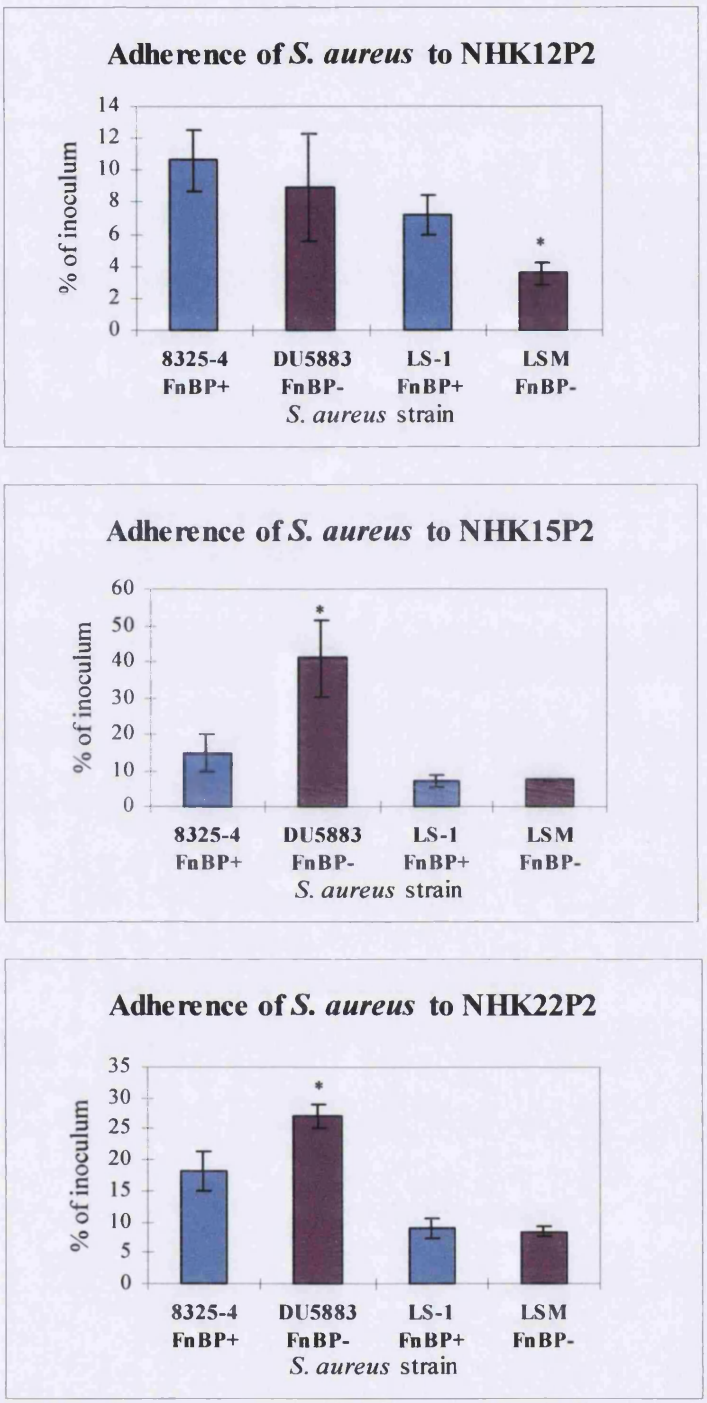


Figure 4-11 The role of *S. aureus* fibronectin-binding protein (FnBP) in *S. aureus* adhesion to primary keratinocyte NHK. Passage two (P2) cultures of NHK from three sources (NHK12, 15, and 22) were incubated with *S. aureus* strains 8325-4 and LS-1 and their respective isogenic mutants DU5883 and LSM (FnBP-) at a concentration of approximately $5x10^7$ cfu for 90 min. Data are the mean of the adherent bacteria ± standard deviation of three replicate cultures.

*, significantly different ($p < 0.05$) from the parental strain.

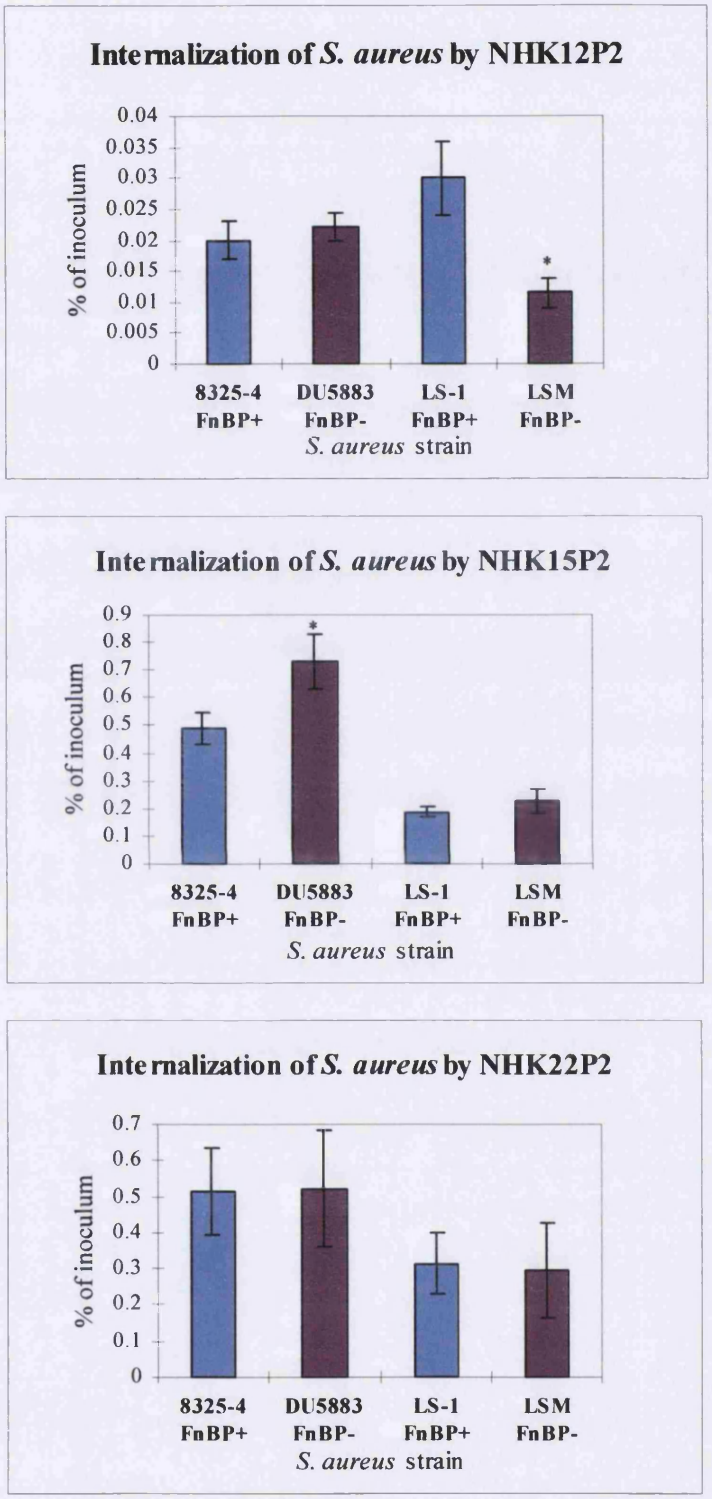
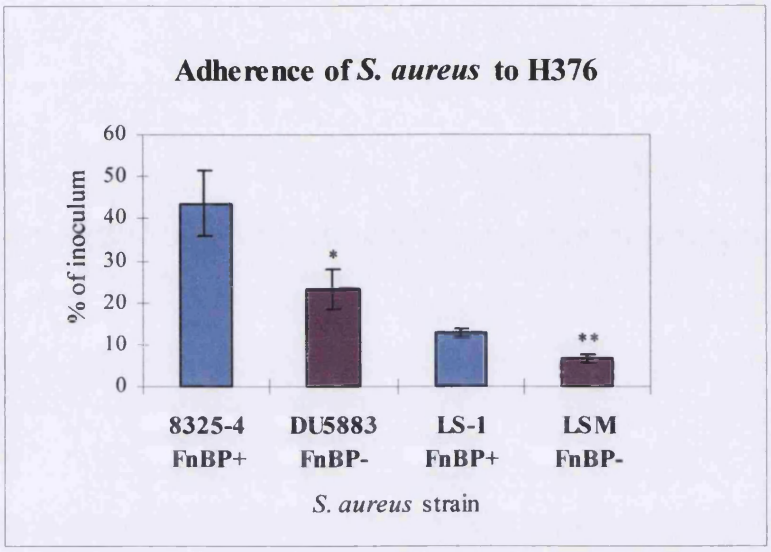
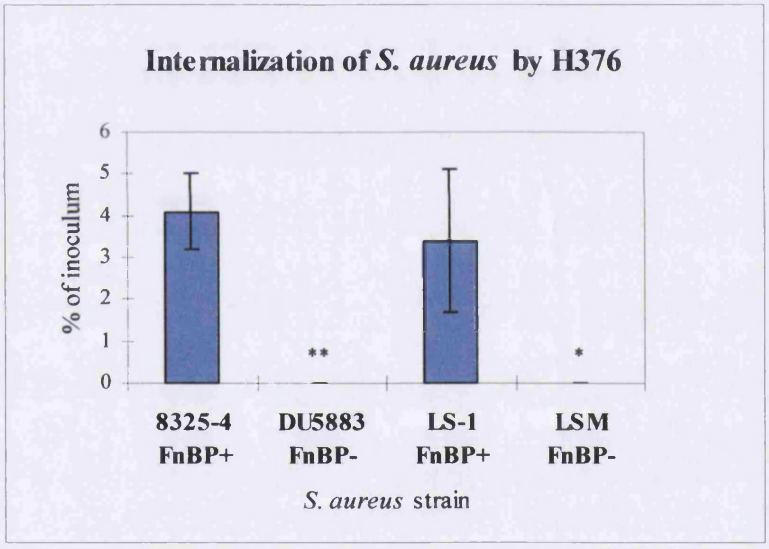


Figure 4-12 The role of *S. aureus* fibronectin-binding protein (FnBP) in *S. aureus* internalization by primary keratinocyte NHK. Passage two (P2) cultures of NHK from three sources (NHK12, 15, and 22) were incubated with *S. aureus* strains 8325-4 and LS-1 and their respective isogenic mutants DU5883 and LSM (FnBP-). Data are the mean ± standard deviation of three replicate cultures.
*, significantly different ($p < 0.05$) from the parental strain.

For comparison with the primary oral keratinocytes, the ability of FnBP-deficient mutants to bind to and be internalized by an oral squamous cell carcinoma line (H376) was examined. Figure 4-13 shows that adherence of FnBP-deficient strains DU5883 and LSM to H376 was markedly decreased. In addition, the FnBP-deficient mutants DU5883 and LSM were not internalized by H376.



(a)



(b)

Figure 4-13 The role of *S. aureus* fibronectin-binding protein (FnBP) in *S. aureus* adhesion (a) and internalization (b) by oral carcinoma line H376. The H376 cells were grown for 2 days in keratinocyte growth medium and incubated with *S. aureus* strains 8325-4 and LS-1 and their respective isogenic mutants DU5883 and LSM (FnBP-) at a concentration of approximately $5x10^7$ cfu for 90 minutes. The number of adherent and internalized bacteria were quantified by plate counting and presented as % of inoculum. Data are the mean ± standard deviation from one of three experiments performed in triplicate with similar results.

*, significantly different ($p < 0.05$) from the parental strain;

**, significantly different ($p < 0.01$) from the parental strain.

4.4 Discussion

Adherence of *S. aureus* to tissue is an important process in colonization and subsequent establishment of infection. Adherent bacteria may remain extracellular or may be internalized into an intracellular compartment. Although *S. aureus* is classically considered an extracellular pathogen, they can be found, and survive, within the cytoplasm of many non-phagocytic cells. In the present study, factors influencing bacterial adhesion to and internalization by human keratinocytes were investigated. These included bacterial vitality and number, infection time, *S. aureus* fibronectin-binding proteins, and expression level of host cell fibronectin-binding integrins.

Immunofluorescence studies in Chapter 3 have shown the presence of FN and $\alpha5\beta1$ integrin on the adherent UP and NHK in cultures. Fibronectin and the $\alpha5\beta1$ integrin are required for the internalization of *S. aureus* by many eukaryotic cells (Sinha *et al*, 1999; Dziewanowska *et al*, 2000; Massey *et al*, 2001).

Microscopic evaluation showed that all three *S. aureus* strains used in the study could adhere and become internalized by immortalized UP keratinocytes and normal oral keratinocytes (NHK). The degree of bacterial adhesion to the cells increased with infection time and inoculum concentration. The results presented here show that adherence of heat-killed *S. aureus* to keratinocytes was less than that of live bacteria. Heat pretreatment at 65°C for 30 min has also shown to strongly inhibit adherence of *Porphyromonas gingivalis* to keratinocytes (Huard-Delcourt *et al*, 1998). It may be that some surface modifications induced by heating and/or recessed bacterial metabolic activity could lead to diminished adherence. Studies in human endothelial cells have shown that *S. aureus* can up-regulate expression of endothelial cell adhesion molecules (*e.g.* E-selectin, ICAM-1 and VCAM-1), and that the viability of the bacteria is required for the induction (Beekhuizen *et al*, 1997; Strindhall *et al*, 2002). The presence of heat-killed *S. aureus* in the intracellular compartment as observed by microscopy after lysostaphin treatment confirms the finding that active metabolic processes from the bacteria are not necessary for internalization (Sinha *et al*, 1999; Kahl *et al*, 2000; Jett and Gilmore, 2002).

The adherence pattern of *S. aureus* varied from strain to strain. NCTC6571 adhered mainly at the periphery of the epithelial sheets of NHK in association with the free edges of the peripheral cells, or in a pericellular distribution around each cell of UP monolayers. Similarly, fibronectin distribution on keratinocytes was predominantly at the perimeter of the UP cells, and at the free edges of peripheral cells of NHK. However, strain 8325-4 adhered on the cell surfaces in a more diffuse distribution pattern. As well as fibronectin, *S. aureus* can also bind to other cell surface matrix proteins such as collagen, laminin, and cell surface glycoproteins (Foster and Höök, 1998). The differences in adhesion pattern with different *S. aureus* strains may be due to differences in surface adhesin production by these strains. In addition, high bacterial count of the adherent NCTC6571 obtained from NHK co-cultures could be related to its large aggregation adherence pattern to the NHK.

The results show that strain LS-1 was internalized by UP keratinocytes to a far higher degree than strain 8325-4 (~ 10-fold higher). *S. aureus* strain LS-1 is commonly used in animal models of septic arthritis because of its highly virulent character (Bremell *et al*, 1991; 1992; Hultgren *et al*, 1998; Sakiniene *et al*, 1999). *S. aureus* 8325-4 is a laboratory strain, however it remains virulent in animal models of infection (Bramley *et al*, 1989; Baddour *et al*, 1994; Gemmell *et al*, 1997). Whilst the results presented in this study show that there were differences between strains 8325-4 and LS-1 in the capacity to become internalized by immortalized UP keratinocytes, no such correlation was found with primary normal keratinocytes or H376 keratinocytes. Studies with *Klebsiella pneumoniae* have also shown cell-dependent invasion in that *Klebsiella pneumoniae* 3091 was highly invasive (determined as % of inoculum) for human T24 bladder and HCT-8 intestinal epithelial cells, but rarely invaded 5637 bladder and INT407 intestinal epithelial cells (Oelschlaeger and Tall, 1997). These data suggest that bacterial internalization is not a simple interaction between human and bacterial cells.

Having established the highly internalization character of strain LS-1 by UP cells compared to strain 8325-4, the number of adherent bacteria (adherent and internalized) was explored. It was found that adherence of strain 8325-4 to UP cells was consistently higher than strain LS-1. These findings demonstrate that the ability

of *S. aureus* strains 8325-4 and LS-1 to adhere to UP cells did not correlate with the levels of internalization. Similar results have been reported with the osteoblast cell line MG-63 (Ahmed *et al*, 2001). It was found that the capacity to bind to immobilized fibronectin and the ability to grow or survive in the intracellular environment did not correlate with the ability of the strain 8325-4 or LS-1 to become internalized by the osteoblasts (Ahmed *et al*, 2001). It was proposed that *S. aureus* might produce virulence factors that either caused an up-regulation in the number of host cell surface receptors responsible for internalization (*e.g.* LS-1) or inhibited its internalization (*e.g.* 8325-4).

In the present study, expression level of one possible host cell receptor for the *S. aureus* internalization, integrin α5β1, was examined. It was found that internalization of the bacteria (both 8325-4 and LS-1) by UP keratinocytes was consistently associated with a slight decrease in the surface expression of β1 integrin subunit, but not the α5 subunit. The decrease in β1 expression suggests a β1-mediated internalization of *S. aureus*. The integrin β1 subunit has been shown to be an important endocytosis factor in bacterial internalization, because mutations in the cytoplasmic domain of the β1 subunit result in the decreased or increased bacterial uptake (Tran Van Nhieu *et al*, 1996). Comparison of the expression level of β1 integrin on UP cells after high uptake of LS-1 with low uptake of 8325-4 showed no differences. These data demonstrate that the decreased surface expression level of the β1 on UP keratinocytes does not correlate with the levels of *S. aureus* internalization. However, differences in the rate of integrin recycling during the uptake of LS-1 and 8325-4 cannot be excluded. *S. aureus* strain LS-1, but not 8325-4, produces TSST-1 toxin that has been reported to stimulate tumour necrosis factor (TNF)-α release by human keratinocytes (Ezepchuk *et al*, 1996). In addition, TNF-α has been shown to induce an increase in the rate of recycling of the internalized α5β1 integrin, resulting in a relatively constant total cell surface expression of the α5β1 on endothelial cells (Gao *et al*, 2000).

Recently *S. aureus* FnBPA and FnBPB have been shown to be required for internalization of this bacterium by human cells such as corneal epithelial cells (Jett and Gilmore, 2002), endothelial cells (Peacock *et al*, 1999) and MG-63 cells (Ahmed

et al, 2001). The results using isogenic mutants with disruption of the *fnb* genes showed that the internalization capacity of the mutants by UP cells was reduced by approximately 80% to 95% when compared to the parental strains. The reduced internalization of FnBP-deficient mutants DU5883 and LSM by UP keratinocytes is probably partly due to a decreased adherent capacity of these strains to UP cells (50% to 68%). Similar reduction in the level of adherence of FnBP-deficient mutant to HaCaT keratinocytes (Mempel *et al*, 1998) and bovine mammary epithelial (MAC-T) cells (Dziewanowska *et al*, 1999) has been reported in which adherence was reduced by 60% and 40% respectively compared to the parental strain. Therefore, it is likely that other surface adhesins and MSCRAMMs compensate for the lack of FN binding by DU5883 and LSM. However, recent reports on the ability of this same isogenic mutant, DU5883, to bind to MG-63 cells (Ahmed *et al*, 2001) and human umbilical vein endothelial cells (Peacock *et al*, 1999) showed that there was more than 90% reduction of its adherence. The differences in adherence to different cell types are likely to be due to differences in matrix molecules expressed by these cells. It has been shown that adherence of mutant strain lacking coagulase, protein A or fibrinogen binding protein or clumping factor A to HaCaT keratinocytes, but not human endothelial cells, was reduced by approximately 50% to 60% (Mempel *et al*, 1998; Peacock *et al*, 1999).

The results obtained from NHK do not support an important role for FnBPs in the efficient internalization of *S. aureus* by primary keratinocytes. The reason for this is not known. However, the difference is not due to difference in the source of the cells because reduced adherence and internalization capacity of FnBP-deficient strains of *S. aureus* were also obtained with oral carcinoma H376 cells as with epidermal UP keratinocytes. It should be noted that primary keratinocytes and immortalized or transformed keratinocytes are different in many ways. Immortalized keratinocytes commonly show a reduced degree of stratification, lower proportion of differentiating cells, and altered integrin expression (Pei *et al*, 1991; Kaur and Carter, 1992; Sugiyama *et al*, 1993). Alteration of keratin (K) expression in UP cells has been reported (Pei *et al*, 1992). They show reduced K16, but increased K18 (associated with simple epithelia) and K13 (associated with non-keratinizing stratified epithelia). In addition, the ability to response to stimuli is also different. For

example, both MMP-2 and MMP-9 are increased in NHK in response to scatter factor, but only MMP-9 is inducible in UP cells (Bennett *et al*, 2000). Whether these differences between the primary and immortalized keratinocytes are responsible for their differences in the interaction with *S. aureus* is to be determined. It is interesting to note that *S. aureus* can bind to K8 (associated with simple epithelia) and K10 (associated with differentiation) (Tamura and Nittayajarn, 2000; O'Brien *et al*, 2002).

In conclusion, the capacity of *S. aureus* to adhere to keratinocytes depended on bacterial strain, vitality, number and infection time. Bacterial metabolic activity was not required for the internalization. In addition, FnBPs of *S. aureus* were important for the internalization. Slight decrease in the expression of β1 subunit in *S. aureus*-infected UP cells suggested the β1-mediated internalization.

Chapter 5

Role of Host Cell Integrins in *S. aureus* internalization and Effects of rFnBPBD1-D4 Protein of *S. aureus* on Human Keratinocyte Adhesion and Motility

5.1 Introduction

Fibronectin (FN) is the major component of the provisional matrix present in the wound space (Yamada and Clark, 1996b), and is often elevated during tissue remodeling and fibrosis. In healing wounds, keratinocytes migrate on a FN-rich granulation tissue matrix. The migrating keratinocytes are highly phagocytic, and can phagocytose FN-coated wound debris and the provisional wound matrix (Takashima and Grinnell, 1984). *Staphylococcus aureus* is a common infecting flora in wound infection. Staphylococcal wound infections can cause delayed healing and may increase risk of death in immunocompromised patients. Although *S. aureus* is an extracellular pathogen, intracellular persistence within keratinocytes (HaCaT) has been demonstrated (von Eiff *et al*, 2001). The *S. aureus* FnBPs contribute to adherence of these bacteria to immobilized FN and to explanted materials (Greene *et al*, 1995). The use of isogenic mutant strains of *S. aureus* with disrupted expression of FnBPs has demonstrated the importance of FnBPs in the adherence to and internalization of *S. aureus* by HaCaT keratinocytes (Mempel *et al*, 1998; 2002), UP keratinocytes and oral squamous carcinoma H376 cells (Chapter 4).

S. aureus strain 8325-4 possesses two closely linked genes, *fnbA* and *fnbB*, that encode surface proteins FnBPA and FnBPB respectively (Signäs *et al*, 1989; Jönsson *et al*, 1991). The FN-binding domains of both FnBPA and FnBPB exhibit ~94% amino acid identity and share homologous repeats with the FN-binding MSCRAMMs expressed by several *Streptococcus* species such as *Streptococcus pyogenes* and *Streptococcus dysgalactiae* (McGavin *et al*, 1993; Joh *et al*, 1994). The structural organization of the FN-binding domains of the FnBPA consists of four tandemly repeat units (D1-D4) and a fifth repeat (Du) located approximately 100 amino acid residues nearer to the N-terminus. Details of the structure and function of FnBPs were reviewed in Section 1.8. Each D repeat unit can bind to the 29-kDa amino-terminal FN fragment (FN-N29-kDa). Synthetic peptides and recombinant

protein fragments of the FN-binding domain (rFnBP) have been reported to inhibit the binding of *S. aureus* to immobilized FN (Raja *et al*, 1990). The rFnBP has also been demonstrated to competitively inhibit internalization of *S. aureus* by human endothelial cells (Massey *et al*, 2001), primary fibroblasts and epithelial 293 cells (Sinha *et al*, 1999). The requirement of host cell FN receptor, integrin α5β1, in the internalization process has also been demonstrated.

Adherent cells polymerize an insoluble FN matrix by assembling cell- or plasma-derived soluble FN into insoluble fibrils (Mosher *et al*, 1992). The first five Type I modules in the N-terminus of FN are essential for FN matrix assembly. The fibronectin-binding domains of the *S. aureus* FnBPs exhibit a high affinity for this region. Competitive binding of the *S. aureus* FnBPs to the N-terminus of FN may decrease the binding and assembly of intact FN by cells, which in turn, affect cell functions such as adhesion, migration and proliferation. In the present study the role of host cell integrin α5β1 in, and the effect of the FN-binding domain of *S. aureus* FnBPs on, *S. aureus* internalization by keratinocytes were determined using anti-integrin function blocking antibodies and a recombinant protein consisting of the D1-D4 repeat units from FnBPB (rFnBPBD1-D4). The role of host cell integrin αvβ6 was determined using carcinoma cell lines expressing different levels of the integrin. The effect of the recombinant protein on keratinocyte adhesion, migration and surface fibronectin polymerization was also investigated.

5.2 Materials and Methods

5.2.1 Keratinocytes

Keratinocytes used in this study were primary human keratinocytes (NHK), derived from explant cultures of oral mucosal tissues, and immortalized UP keratinocyte cell line. Three oral squamous carcinoma cell lines: H357, VB6 and C1, were also used in some experiments. VB6 and C1 cell lines are generated from H357 which is an αv-negative cell line (Prime *et al*, 1990; Sugiyama *et al*, 1993). The H357 cell line was transfected with αv cDNA to create V3 cells, which predominantly express the αvβ5 integrin (Jones *et al*, 1996). The V3 cell line was retrovirally infected with β6 cDNA creating the VB6 cell line, which has high αvβ6

integrin expression (Thomas *et al*, 2001a). The C1 cell line is a null transfectant control cell line for the generation of the VB6 cells. Accordingly, VB6 and C1 cells express high and low surface levels of αvβ6 integrin respectively. All keratinocytes were maintained in KGM as described in Section 2.2.

5.2.2 *S. aureus* Strains and Culture Condition

S. aureus strain 8325-4 was used in all bacterial internalization assays in this Chapter. Strains NCTC6571 and LS-1 were included in some experiments. All *S. aureus* strains were grown in Todd Hewitt broth and used at exponential growth phase, as described in Section 2.9.3.

5.2.3 Expression and Purification of Recombinant FnBPBD1-D4 Protein

The recombinant protein was prepared and expressed as a histidine-tagged (6xHis-tagged) protein. Cell lysates of *Escherichia coli* strain M607 containing the rFnBPBD1-D4 protein were prepared as described in Section 2.13.1. The 6xHis-tagged rFnBPBD1-D4 protein was purified by Ni-NTA affinity chromatography under native condition as described in Section 2.13.2. The eluted fractions were analyzed by SDS-PAGE and a single protein band with molecular weight of approximately 35 kDa was obtained (Figure 2-3). The proteins were pooled and dialysed against PBS. The protein concentration was determined by measuring the absorbance at 280 nm, using an absorbance of 0.220 for 1 mg/ml.

5.2.4 Determination of the Role of Integrins Expressed on Keratinocytes in Adhesion and Migration Assays on Immobilized Fibronectin

Adhesion and migration blocking experiments were used to determine if the FN-binding integrins (α5β1 and αvβ6) expressed on UP and NHK were functional. Cell adhesion assays were performed in FN-coated 96-well plates as previously described in Section 2.5. The haptotactic cell migration assays were performed using FN-coated Transwell® inserts in 24-well plates as previously described in Section 2.6. For blocking experiments, suspensions of UP and NHK in SFM (for the adhesion assays) or migration buffer (for the migration assays) were pre-incubated with function blocking antibodies against α5 (P1D6), β1 (P4C10 for UP and P5D2

for NHK), αv (L230) and αvβ6 (10D5) integrins (Table 5-1) for 15 min at room temperature before plating. Cells, in the presence of the antibodies, were allowed to adhere for 2 hours or migrate for 4 hours. Cells incubated without antibody, for adhesion assays, or with mouse IgG2a negative control (1:50; Dako Ltd), for migration assays, served as controls. Data are presented as percentage of controls.

Table 5-1 Antibodies used in cell adhesion and migration blocking experiments.

Antibody	*Clone*	*Dilution*	*Source*
Mouse IgG2a	-	1:50 (2 μg/ml)	Dako Ltd
α5 integrin	mAb P1D6	1:10	Dako Ltd
β1 integrin	mAb P4C10	1:100 (10 μg/ml)	Chemicon Intl Ltd
	mAb P5D2	1:50 (20 μg/ml)	Chemicon Intl Ltd
αv integrin	mAb L230	20 μg/ml	Gift from JF Marshall
αvβ6 integrin	mAb 10D5	1:50 (20 μg/ml)	Chemicon Intl Ltd

5.2.5 Effect of Anti-integrin α5β1 and rFnBPBD1-D4 Protein on Internalization of *S. aureus* by UP and NHK

Bacterial internalization assays were conducted as described in Section 2.10 with the use of exponential phase cultures of *S. aureus* strain 8325-4 at a final concentration of $5x10^7$ cfu/ml/well. To examine the role of integrin α5β1 and rFnBPBD1-D4 protein in *S. aureus* internalization by keratinocytes, antibodies (300 μl) and rFnBPBD1-D4 (500 μl) were pre-incubated with keratinocyte cultures for 30 min before addition of the bacteria and remained present during internalization assays. The keratinocytes were co-incubated with the bacteria for 90 min at 37°C. The antibodies used were the function blocking anti-integrins α5 (P1D6, 1:10), α5β1 (JBS5, Chemicon Intl Ltd, 8 μg/ml) and β1 (P4C10 at 8 μg/ml for UP, P5D2 at 20 μg/ml for NHK). Control wells contained neither antibody nor the recombinant protein.

5.2.6 The Role of αvβ6 Integrin in the Internalization of *S. aureus* by Keratinocytes

To determine the role of host cell αvβ6 integrin in the internalization of *S. aureus* by keratinocytes, the ability of *S. aureus* to be internalized by three squamous carcinoma cell lines (H357, VB6 and C1) expressing different surface levels of the αvβ6 integrin were compared. H357 cells are αv negative. VB6 and C1 cells express high and low levels of αvβ6 integrin respectively. H357, VB6 and C1 cells express similar surface levels of α5β1 integrin as determined by flow cytometry (Thomas *et al*, 2001a; 2001b). Keratinocytes were grown to confluence for 2 days in KGM and bacterial internalization assays were performed as described in Section 2.10 with the use of exponential phase *S. aureus* strains NCTC6571, 8325-4 and LS-1 at a concentration of $5x10^7$ cfu/ml. Keratinocytes were co-incubated with *S. aureus* for 60 or 90 minutes.

5.2.7 Effects of rFnBPBD1-D4 Protein on Keratinocyte Adhesion to and Migration on Immobilized Fibronectin

Cell adhesion assays were performed in 96-well plates coated with 10 μg/ml FN as previously described in Section 2.5 with the following modifications. Briefly, after the wells were coated with FN and blocked with 0.5%BSA-PBS, 50 μl of various concentrations of rFnBPBD1-D4 protein diluted in SFM were added in each well, in triplicate, and incubated at 37°C for 1 hour and remained present for the duration of the adhesion assays. Keratinocytes at $3x10^4$ cells in 50 μl were then added to each well and were allowed to adhere for 2 hours. Control wells were not treated with or did not contain the recombinant protein.

The haptotactic cell migration assays were performed using Transwell® inserts coated with 10 μg/ml FN as previously described in Section 2.6 with some modification. Briefly, the underside of the membrane inserts were double-coated with rFnBPBD1-D4 protein by transferring the inserts from FN-containing wells into new wells containing 250 μl of 10 or 100 μg/ml rFnBPBD1-D4 protein diluted in migration buffer, in triplicate, and incubated at 37°C for 1 hour. The inserts were transferred into new wells for blocking with migration buffer at 37°C for another

hour. Migration buffer was then removed from the upper compartment of the Transwell® and replaced with 100,000 cells in 100 µl of migration buffer. The inserts were then returned to the recombinant protein-containing wells in which additional migration buffer (350 µl) was added to a final volume of 600 µl. Cells were allowed to migrate for 4 hours. Control Transwell® inserts were not treated with the recombinant protein. Data (numbers of migrated cells per field) were presented as a percentage of control.

5.2.8 Wounding Assay

UP cells were used in the wounding assays. The cells were plated in a 24-well plate at a concentration of 70,000 cells per well in KGM, and were confluent within 2 days. A cross-mark scraping was created using a sterile 200-µl pipette tip. The scrape-wounded monolayers were washed twice with SFM containing 0.2% BSA (SFM-0.2%BSA), and 500 µl per well of the SFM-0.2%BSA with or without rFnBPBD1-D4 at 100 µg/ml was added to the wells in duplicate. The extent of migration was determined after 20 hours of wounding. To determine the migrating areas, phase contrast micrographs at low magnification (10x) were taken after wounding at 0 and 20 hours from each line of the cross-mark wounding (4 wound areas per well). A region of interest representing the whole wounded area on the micrograph was drawn manually and the area was measured automatically using the Universal Leica Qfluoro Imaging software program. The migrating area (arbitrary units) was the area after substraction of the wounded area at 20 hours from that at 0 hour. Cell migration in the absence of the recombinant protein served as control. Data (migrated areas) are presented as a percentage of control.

5.2.9 Cell Cycle Progression Using BrdU Assay

To investigate cell growth during wound closure, BrdU incorporation was used to quantify the fraction of S phase cells from wounded areas. Briefly, UP cells were prepared as described in the wounding assay on glass coverslips in a 24-well plate. After wounding, the wounded monolayers were washed twice with SFM-0.2%BSA, and 500 µl per well of the SFM-0.2%BSA, with or without the recombinant protein rFnBPBD1-D4 at 100 µg/ml, was added to the wells. The

fraction of S-phase cells was determined at 1 and 20 hours after wounding by addition of 5-Bromo-2'-deoxy-uridine (5-BrdU; Sigma®) to a final concentration of 10 μM one hour before the indicated time. Continuous exposure of the UP cells to 5-BrdU was also performed by adding the BrdU immediately after wounding at a final concentration of 20 μM. After 20 hours of continuous exposure or 1 hour of single time point BrdU pulse, cells were fixed in ice-cold absolute methanol for 10 min, rinsed twice in PBS, and permeabilized with 0.25% TritonX-100 for 15 min at room temperature. The cells were gently washed three times in PBS, then incubated with 2 M hydrochloric acid (HCl) for 20 min to denature the double-stranded DNA. The cells were then washed three times with DMEM-HEPES (Gibco™) and PBS, and incubated in 0.1 M sodium borate ($Na_2B_4O_7$) for 20 min to neutralize the HCl. After removal of the $Na_2B_4O_7$ without washing, the cells were incubated with anti-BrdU (Alexa Fluor® 488 conjugate, Molecular Probes Europe BV, Leiden, The Netherlands) at 20 μg/ml in 0.1 M $Na_2B_4O_7$ for 1 hour. After three washes in PBS, the cells were double stained with 0.01 μg/ml 4,6-Diamidino-2-phenylindole (DAPI; Sigma®) to visualize cell nuclei. The cell containing coverslips were washed three times in PBS and mounted on glass slides using glycerol-PBS mounting medium (Citifluor™). The staining procedure was performed at room temperature. Approximately 750 to 1,000 cells along the wound edge were counted. The number of BrdU positive cells was expressed as a percentage of the total number of cells stained with DAPI.

5.2.10 Effect of rFnBPBD1-D4 Protein on Endogenous Fibronectin Matrix Polymerization by UP Keratinocytes

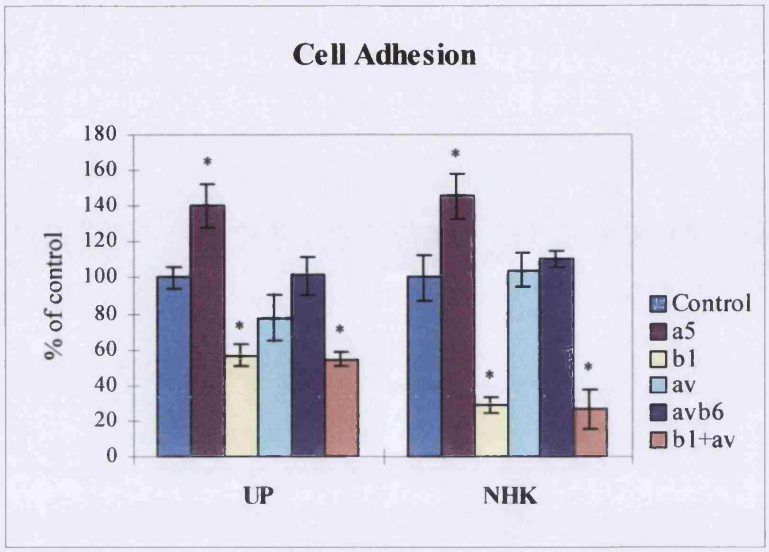
Subconfluent UP cultures were washed with PBS, harvested by trypsinization and suspended in Defined Keratinocyte-SFM (Gibco™). The suspensions of UP keratinocytes at a concentration of $7x10^4$ cells per well were plated on glass coverslips coated with 10 μg/ml human plasma fibronectin in 24-well plates in the presence or absence of 100 μg/ml rFnBPBD1-D4 protein. In some experiments, the rFnBPBD1-D4 protein was replaced by 100 μg/ml 30 kDa amino-terminal fragment of FN (Sigma®). The cells were then incubated at 37°C in 5% CO_2 for 24 h. Cell-containing glass coverslips were then washed twice with PBS and fixed with 4%

paraformaldehyde for 10 min. Indirect immunocytochemistry was performed without permeabilization as described in Section 2.7 to detect endogenous cellular fibronectin (cFN). The cFN was detected using monoclonal antibody IST-9 (10 μg/ml), which recognizes the EDA type III module which is present in cellular but not plasma fibronectin (less than 1%) (ffrench-Constant, 1995).

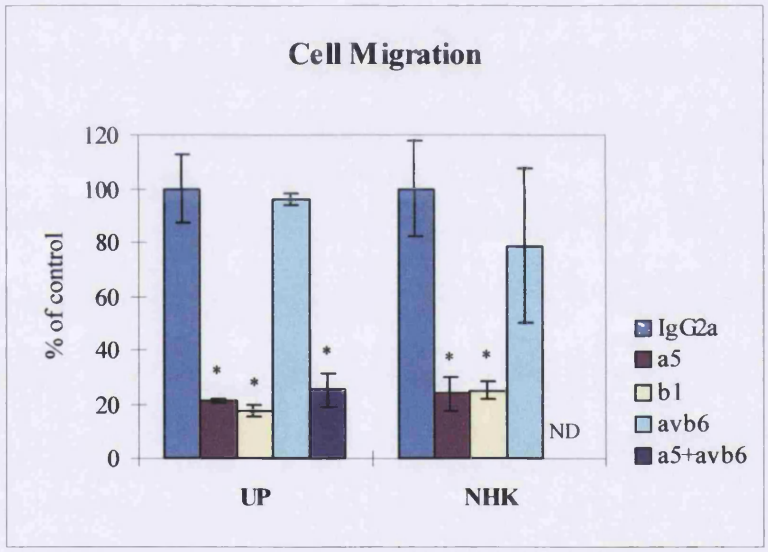
5.3 Results

5.3.1 Functional Activities of Integrins α5β1 and αvβ6 in UP and NHK

Using immunofluorescent microscopy (Chapter 3), the presence of FN and integrins α5β1 and αvβ6 on the surface of the adherent keratinocytes (UP and NHK) used in the bacterial internalization assays was confirmed. Before determination of the effect of anti-integrin antibodies on *S. aureus* internalization, the functional activities of the integrins were assessed by blocking experiments on FN-coated culture plates. The results are shown in Figure 5-1. Antibodies directed against αv and αvβ6 had no effect on the adhesion and migration of either UP or NHK. Antibody directed against the β1 subunit significantly inhibited cell adhesion to (40% inhibition for UP and 70% for NHK) and migration on (80% inhibition for both UP and NHK) fibronectin. No additional inhibition of adhesion was obtained using a combination of anti-β1 and anti-αv. Using a specific antibody against the α5 subunit to block function of the α5β1 integrin showed no inhibition of the cell adhesion. In contrast, it significantly enhanced cell adhesion to fibronectin for both UP and NHK while inhibiting cell migration (80% inhibition for both UP and NHK). These data demonstrate that α5β1, but not αvβ6 integrins, contribute the major role in UP and NHK adhesion to and migration on fibronectin.



(a)



(b)

Figure 5-1 Effects of inhibitory anti-integrin antibodies on the adhesion to (a) and migration on (b) fibronectin of UP and NHK. Keratinocytes were plated in the presence or absence of the blocking antibodies and allowed to adhere for 2 hours or migrate for 4 hours. Data are mean ± standard deviation of a representative experiment performed in triplicate.

*, significantly different from the control, $p \leq 0.01$; ND, not determined.

5.3.2 Integrin α5β1 Mediates Internalization of *S. aureus* by Immortalized Keratinocyte Cell Lines But Not by Primary Keratinocytes

To investigate the role of integrin α5β1 in the uptake of *S. aureus* by keratinocytes, bacterial internalization assays were performed after pre-incubation of UP, H357 and NHK cells with function blocking anti-integrin α5β1 antibodies (Figure 5-2). Internalization of *S. aureus* strain 8325-4 by UP and H357 was reduced by more than 50% in the presence of the antibody directed against α5β1 integrin (clone JBS5). However, the JBS5 did not alter the internalization of *S. aureus* by NHK. Antibodies directed against α5 (P1D6) and β1 subunits had no inhibitory effect on *S. aureus* internalization. In contrast, P1D6 enhanced *S. aureus* internalization by all the keratinocyte cell lines tested including NHK.

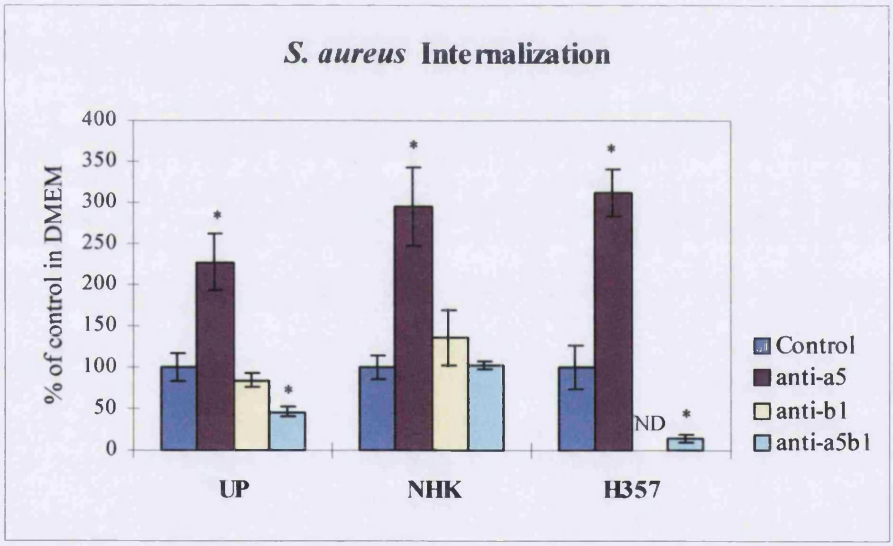
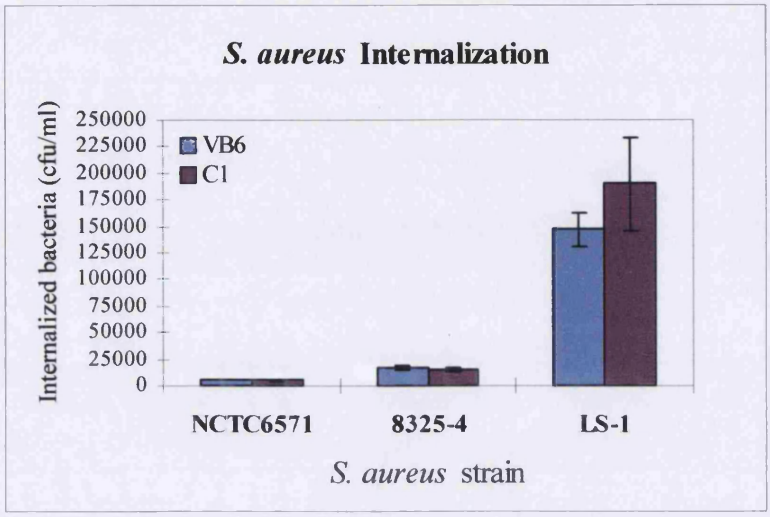


Figure 5-2 Effect of anti-integrin antibodies on internalization of *S. aureus* strain 8325-4 by UP, NHK and H357 cells. Internalization assays were performed after pre-incubation of keratinocytes with anti-α5 (clone P1D6, 1:10), anti-β1 (clone P4C10 at 8 μg/ml for UP and clone P5D2 at 20 μg/ml for NHK) or anti-α5β1 (clone JBS5, 8 μg/ml). Control wells contained no antibody. Data are mean ± standard deviation of a representative experiment performed in triplicate.

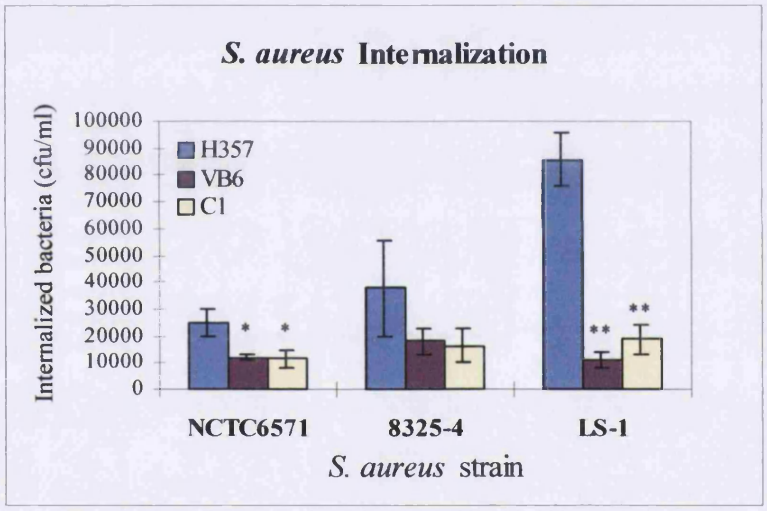
*, significantly different from the control, $p \leq 0.01$; ND, not determined.

5.3.3 Integrin αvβ6 Does Not Mediate *S. aureus* Internalization

Integrin αvβ6, a fibronectin receptor, is expressed during wound healing, in oral squamous cell carcinoma and in normal keratinocytes in culture. To determine the role of host cell αvβ6 integrin in *S. aureus* internalization, the ability of *S. aureus* to be internalized by VB6 and C1 cells were compared. Internalization levels of *S. aureus* strain NCTC6571, 8325-4 or LS-1 by VB6 and C1 were similar (Figure 5-3a). This suggests that αvβ6 integrin is not the main receptor on keratinocytes that mediate the internalization of *S. aureus*. It is then postulated that the restoration of αvβ6 integrin in an αv-negative cell line would be unlikely to have marked effect on *S. aureus* internalization. To test this hypothesis, internalization of *S. aureus* by H357 and VB6 cells were compared. It was found that the uptake of *S. aureus* by H357 was much higher than that by VB6 cells (Figure 5-3b). There were no differences in the adherent levels of *S. aureus* between H357, VB6 and C1 cells between the *S. aureus* strains tested (data not shown).



(a)



(b)

Figure 5-3 The role of integrin αvβ6 in the internalization of *S. aureus* by keratinocytes. (a) Comparison between high (VB6) and low (C1) αvβ6 expressed cell lines. Cells were co-incubated with $5x10^7$ cfu/ml *S. aureus* for 90 min.
(b) Comparison between αv-negative H357 and VB6 and C1 cells. Cells were co-incubated with $5x10^7$ cfu/ml *S. aureus* for 60 min. Data are presented as mean ± standard deviation of a representative experiment performed in triplicate.
*, significantly different ($p < 0.05$) from the H357;
**, significantly different ($p < 0.01$) from the H357.

5.3.4 *S. aureus* FnBP is Important for the Internalization of *S. aureus* by UP Cells But Not by NHK

Previous studies in Chapter 4 using mutant strains of *S. aureus* have shown that loss of FnBPs did not alter the capacity of *S. aureus* to be internalized by NHK (Figure 4-12). However, internalization of *S. aureus* lacking FnBPs by immortalized keratinocyte (UP) and carcinoma cell line (H376) was reduced by more than 90% compared to the wild-type strains (Figures 4-10 and 4-13), indicating a selective effect of FnBPs in internalization of *S. aureus* by keratinocyte cell lines. To investigate the selective role of FnBPs in *S. aureus* internalization further, the effect of a soluble recombinant protein encompassing the D1-D4 repeat units of FnBPB (rFnBPBD1-D4) on *S. aureus* internalization was tested. Internalization of *S. aureus* by UP cells was markedly reduced by approximately 80% in the presence of rFnBPBD1-D4, whereas there was no effect in the internalization by NHK even at higher concentrations (Figure 5-4).

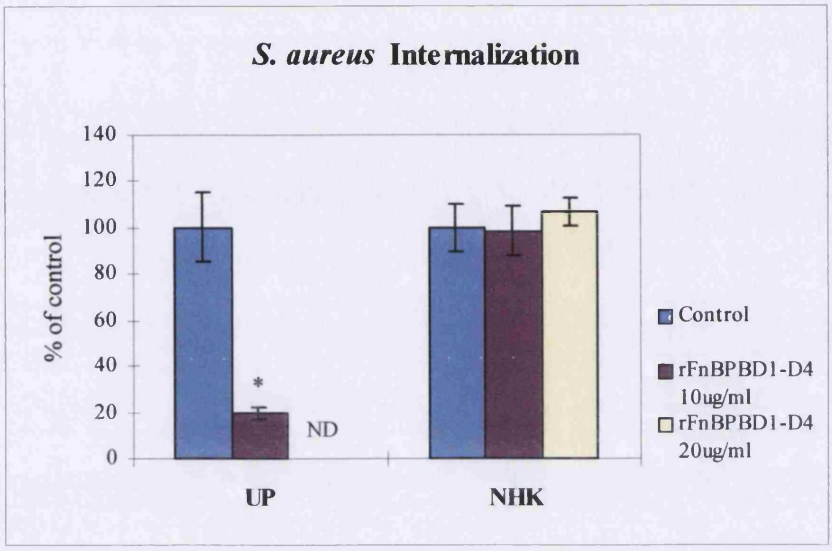


Figure 5-4 Effect of the recombinant D1-D4 repeat units of FnBPB (rFnBPBD1-D4) on *S. aureus* internalization. Internalization assays were performed in the presence or absence (control) of 10 μg/ml or 20 μg/ml rFnBPBD1-D4 protein. Data are presented as mean ± standard deviation of a representative experiment performed in triplicate. Repeat experiments showed similar results. *, significantly different from the control, $p < 0.001$; ND, not determined.

5.3.5 Effect of rFnBPBD1-D4 Protein on Keratinocyte Adhesion

To determine the effect of rFnBPBD1-D4 protein on cell adhesion to fibronectin, keratinocytes were plated onto FN-coated tissue culture plates double-coated with increasing concentrations of the recombinant protein which were also present during the adhesion assays. As shown in Figure 5-5, rFnBPBD1-D4 had no effect on the adhesion of both UP and NHK.

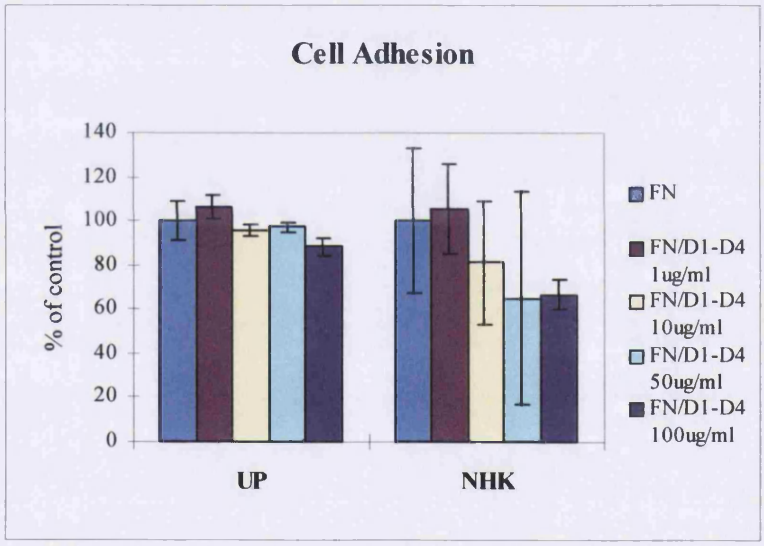


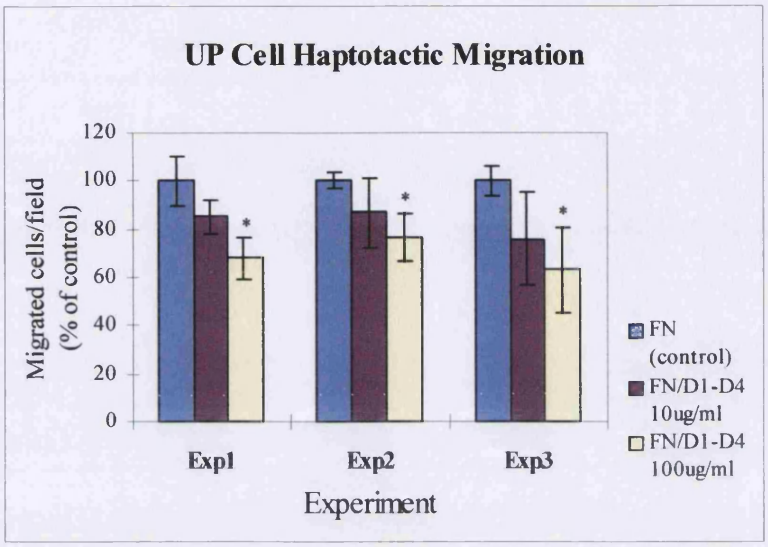
Figure 5-5 The recombinant D1-D4 repeat unit of FnBPB (rFnBPBD1-D4) does not affect the adhesion of UP cells or NHK to fibronectin. Adhesion assays were performed in fibronectin (FN)-coated 96-well plates double coated with various concentrations of the recombinant protein. Control wells were coated with FN only. Data are presented as mean ± standard deviation of a representative experiment performed in triplicate.

5.3.6 Effects of rFnBPBD1-D4 Protein on UP and NHK Migration and UP Cell Cycle Progression

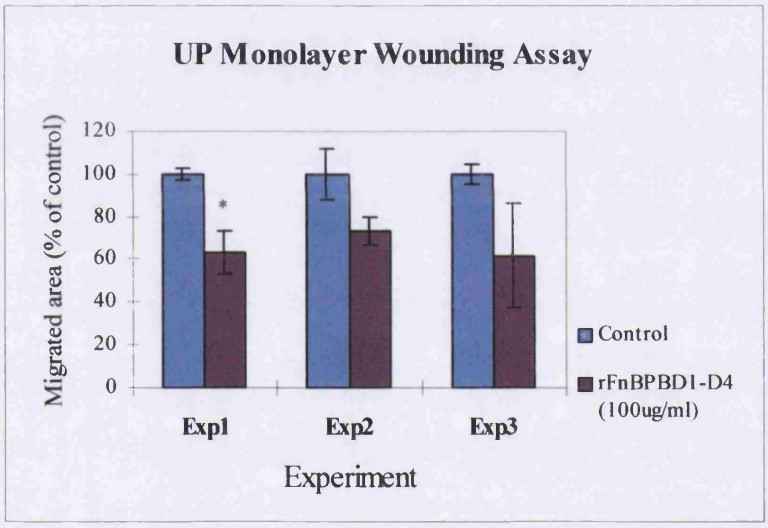
Two methods were used to study the effect of rFnBPBD1-D4 on cell motility: haptotactic cell migration assays and wounding assays. The haptotactic assay was used to determine single-cell migration along an adhesion gradient within a short period of 4 hours. The wounding assay was used to mimic cell migration during wound healing and was determined at 20 hours. UP cells migrated less well on co-immobilized rFnBPBD1-D4 and FN substrate than on FN alone (Figure 5-6a). There was a relative reduction of 17% (SD = 5.88, $p = 0.036$) and 31% (SD = 7.00, $p = 0.017$) over three independent experiments for immobilized rFnBPBD1-D4 protein at 10 and 100 μg/ml respectively. Migration of NHK on the co-immobilized rFnBPBD1-D4 and FN substrate was not different from that of control cells on FN alone (data not shown, see Appendix C).

Monolayers of UP cells were scrape-wounded and incubated in SFM-0.2%BSA containing 100 μg/ml rFnBPBD1-D4 protein. By 20 hours, cells migrated into the wounded areas as a contiguous sheet in the same way as the cells in control wells. The morphology of the cells in the presence of rFnBPBD1-D4 was not different from the controls (data not shown). A consistent decrease in UP cell migration in the presence of the recombinant protein, measured as migrated area, was observed (Figure 5-6b). There was a relative reduction of 33% (SD = 5.34, $p = 0.009$) over three independent experiments. To determine if cell migration during 20 hours of wound closure of UP cell monolayers was dependent on proliferation, the cellular proliferative index was assessed in both control and rFnBPBD1-D4 protein-treated wounded monolayer cultures. BrdU incorporation was used to quantify the fraction of S phase cells in scrape-wounded monolayers. Continuous exposure to BrdU provides an estimate of the fraction of non-cycling cells, and pulse-chase experiments provide quantitative measures of cell progression. Differences in BrdU uptake in rFnBPBD1-D4-treated cells and non-treated controls were not observed. Approximately 24% (SD = 3.37) of the nuclei of cells along the wound edges stained with BrdU at the first hour post-injury. Continuous exposure to BrdU for 20 hours showed a slight increase in BrdU uptake to 28% (SD = 0.59) and 29% (SD = 4.73) in

non-treated controls and rFnBPBD1-D4-treated cells respectively. A single time point BrdU pulse at 20 hours showed a reduction of BrdU-stained cells to approximately 17.6% (SD = 1.8) for both rFnBPBD1-D4-treated and non-treated cells. These data indicate that the proliferation rate of UP cells in serum-free conditions is low and the proportion of the cells entering cell cycle process is decreased after 20 hours. Addition of the recombinant protein at the concentration that decreased cell migration had no effect on BrdU uptake.



(a)

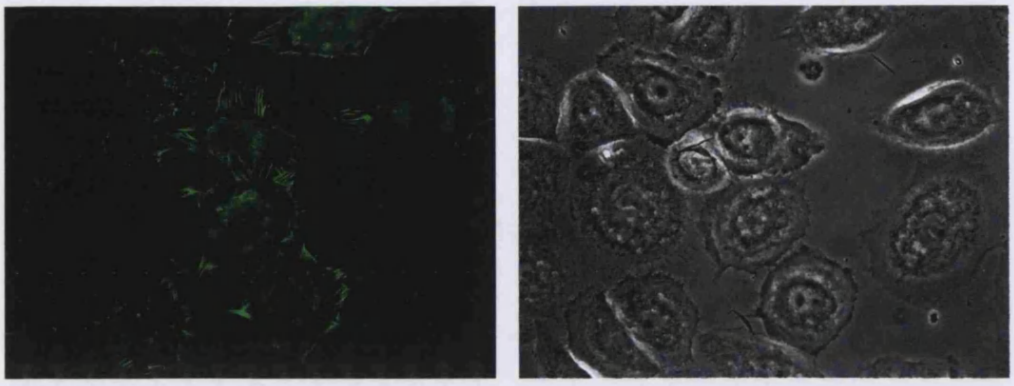


(b)

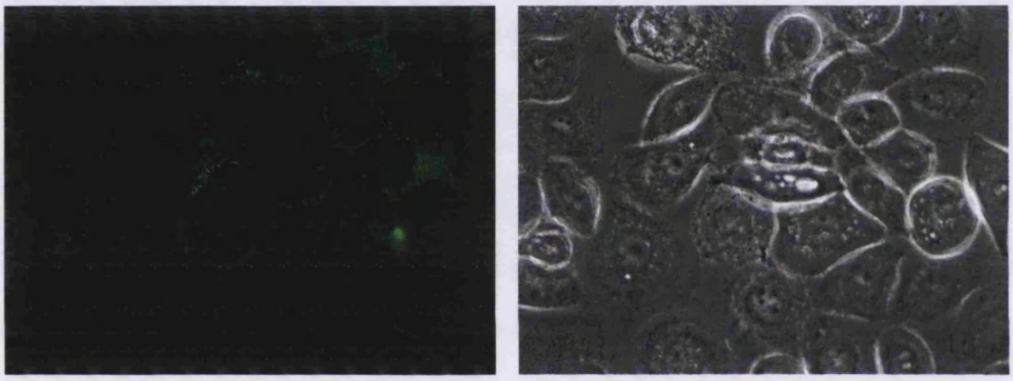
Figure 5-6 Effect of the recombinant D1-D4 repeat units of FnBPB (rFnBPBD1-D4) on UP cell migration. (a) Haptotactic migration. Cells were plated into fibronectin-coated Transwell® inserts double-coated with 10 or 100 μg/ml rFnBPBD1-D4 protein and the cells were allowed to migrate for 4 hours. Control wells were not double-coated with the recombinant protein. (b) Wounding assay. Confluent cell monolayers were scrape-wounded and the cells were allowed to move into the wound for 20 hours in the absence (control) or presence of 100 μg/ml rFnBPBD1-D4 protein. Results from three separate experiments are shown. Data are presented as mean ± standard deviation. *, $p < 0.05$.

5.3.7 Effect of rFnBPBD1-D4 Protein on Endogenous Fibronectin Matrix Polymerization

Since the D repeat units of *S. aureus* FnBPs exhibit high affinity to the amino-terminal of FN, the rFnBPBD1-D4 protein was tested for its ability to affect assembly of endogenous fibronectin. UP cells plated for 24 hours on a FN-coated surface in the serum-free defined medium, attached, spread and formed epithelial colonies (Figure 5-7). Staining for endogenous FN using IST-9 without permeabilization showed that the FN was produced by UP cells and was organized predominantly at the periphery of cells and between cells along the cell filopodia. In the presence of rFnBPBD1-D4, UP cells also attached, spread and formed colonies similar to the control cultures in the absence of the protein. However, most of the cellular FN matrix on the cell surface was absent, and replaced with punctate deposits of endogenous FN on the background substratum (Figure 5-7b). The absence of cell-associated FN matrix was also observed similarly in the presence of 30 kDa amino-terminal FN fragment (100 μg/ml, data not shown), which has been shown to inhibit FN assembly (Akimov and Belkin, 2001; Kamiya *et al*, 2002).



(a)



(b)

Figure 5-7 Effect of the recombinant D1-D4 repeat units of *S. aureus* FnBPB (rFnBPBD1-D4 protein) on endogenous fibronectin (FN) matrix polymerization. UP keratinocytes were seeded at $7x10^4$ cells/well on glass coverslips coated with 10 μg/ml plasma FN in 24-well plates in serum-free defined medium in the absence (a) or presence (b) of 100 μg/ml rFnBPBD1-D4 protein. Cells were incubated for 24 h at 37°C, and then processed for immunofluorescence as indicated in the Materials and Methods section. These selected micrographs are representative of multiple fields. Cells were photographed at the same magnification and using the same exposure times. Original magnification: x630.

5.4 Discussion

Cell migration is a dynamic process that involves the generation of adhesive force and traction followed by the adhesive release. Most studies have shown that anti-integrin function blocking antibodies inhibited adhesion of cells to substrate resulting in reduction in cell migration (Lange *et al*, 1994; 1995; Huang *et al*, 1998). In the present study, anti-integrin function blocking antibody to the β1 subunit (P4C10 or P5D2) which blocks all β1 integrins, inhibited cell adhesion to FN. This is in agreement with previous studies using P5D2 and human epidermal keratinocytes (Levy *et al*, 2000). Blocking antibody to the α5 subunit (P1D6) enhanced adhesion of both UP and NHK to the FN substrate, but it inhibited cell migration. A mechanistic interpretation is that very weak adhesions result in poor traction, whereas very strong adhesions may not release rapidly, resulting in inhibited migration. These data are consistent with the concept that intermediate adhesive strength is important for optimal migration (DiMilla *et al*, 1993; Huttenlocher *et al*, 1995; 1996). The absence of a role for integrin αvβ6 in the adhesion and migration of UP and NHK may be due to its low expression level as assessed by indirect immunofluorescence and flow cytometry (Chapter 3).

The interaction between *S. aureus* and host cells has been shown to occur through a bridging model where FN is bound by *S. aureus* FnBPs as well as host cell integrin. Using the mouse fibroblast cell line GD25 lacking expression of the β1 family of integrins, the internalization of *S. aureus* was reduced by ~97% compared to the complemented cell line expressing β1 integrin (Fowler *et al*, 2000). The function blocking anti-β1 antibody (P4C10) has been reported to inhibit uptake of *S. aureus* by HEp-2 cells, but failed to block uptake by MAC-T cells (Dziewanowska *et al*, 2000). The different responses to P4C10 blocking may depend on the tissue source. Internalization of *S. aureus* by 293 cells (Sinha *et al*, 1999) and HUVEC (Massey *et al*, 2001) was also reduced by more than 50% in the presence of anti-α5β1 antibody (JBS5). Previous results from FnBP-deficient mutants and rFnBPBD1-D4 experiments have shown that FnBPs were important for internalization of *S. aureus* by immortalized keratinocyte cell lines. In the present study, the role of integrin α5β1 in the internalization of *S. aureus* by keratinocytes

was assessed using three different blocking antibodies: anti-α5 (P1D6), anti-β1 (P4C10) and anti-α5β1 (JBS5). It was found that uptake of *S. aureus* by UP cells was reduced by more than 50% in the presence of JBS5, whereas P4C10 had no effect and P1D6 enhanced the internalization. Similar results were obtained with the oral squamous carcinoma cell line H357. The different effects of P1D6, P4C10 and JBS5 on the uptake of *S. aureus* may be due to differences in their functional properties. P1D6 blocks the recognition by α5β1 of the PHSRN synergy sequence in FN but does not block the recognition of RGD (Mould *et al*, 1997). JBS5 blocks the recognition of both the synergy and RGD sequences in FN (Mould *et al*, 1997; 1998). Anti-β1 mAbs or bivalent cations inhibit the binding of JBS5 to α5β1 but do not affect the binding of P1D6 (Mould *et al*, 1998). P4C10 blocks the recognition of the RGD sequence in FN (Mould *et al*, 1997).

It has been shown that the integrin-mediated internalization following bacterial attachment to the cell is primarily dependent on the affinity of the bacterial ligand for the integrin (Tran Van Nhieu *et al*, 1993). Previous results in this Chapter have shown that integrin α5β1 is a major functional receptor on UP keratinocytes and NHK for FN. The high affinity interaction of integrin α5β1 with the central cell binding domain of FN requires both the RGD sequence in the tenth type III repeat and the synergy sequence in the ninth type III repeat (Obara and Yoshizato, 1995; Mould *et al*, 1997). In addition, the activity of JBS5 is reported to involve some direct competitive inhibition (Mould *et al*, 1997). Accordingly, competitive inhibition of the binding at both RGD and synergy sites by JBS5 results in low affinity binding of the integrin with FN which in turn decreases internalization of the associated *S. aureus*.

S. aureus FnBPs bind to FN at the amino-terminus with high affinity of a nanomolar range (Huff *et al*, 1994; Joh *et al*, 1994). Conformational changes of the FN may occur upon binding with the *S. aureus* FnBPs (in excess) and increase FN-integrin binding affinity. In addition, the recognition sites on α5β1 for some blocking antibodies (*e.g.* mAb13, P1D6 and P4C10) are attenuated as a consequence of ligand occupancy (Mould *et al*, 1996; 1997). It is possible that *S. aureus* FnBPs-FN ligand occupancy of α5β1 attenuates the binding of P4C10 and its inhibitory effect on *S.*

aureus internalization since the cytoplasmic domain of the β1 subunit regulates bacterial internalization (Tran Van Nhieu *et al*, 1996; Gustavsson *et al*, 2002). For P1D6, although it blocks the binding of FN at the synergy site, it seems to increase cell adhesiveness to FN in the present study. In addition, P1D6 can stimulate signaling proteins, but this is dependent on the degree of integrin aggregation (Symington, 1995). It is possible that signaling generated by the α5 subunit stimulates activity of the cytoplasmic domain of the β1 and activates internalization in an outside-in signaling pathway. It has been previously demonstrated that the signals generated by binding of FN to the α5β1 integrin receptor on monocytes are transduced by the α5 subunit cytoplasmic domain that leads to increased activity of the α2β1 receptor, without modification of its expression, and increased monocyte adhesion to the collagen substrate (Pacifici *et al*, 1994).

Integrin αvβ6, a fibronectin receptor, is not detectable on normal epithelium, however, it is highly expressed in the migrating basal keratinocytes during wound healing (Breuss *et al*, 1995; Clark *et al*, 1996; Haapasalmi *et al*, 1996). The up-regulated integrin may be utilized by wound-contaminated bacteria for tissue adherence and invasion. A possible role of αvβ6 in the adhesion or internalization of *S. aureus* by keratinocytes was therefore investigated using high (VB6) and low (C1) αvβ6 expressing oral squamous carcinoma cell lines. There were no differences in the adhesion or internalization level of *S. aureus* between VB6 and C1 when they were challenged with either strain NCTC6571, 8325-4 or LS-1. This suggests that αvβ6 integrin is not an important receptor on keratinocytes for the mediation of adhesion or internalization of *S. aureus*. Interestingly, the level of internalized *S. aureus* by the αv-negative parental cell line H357 was much higher than both VB6 and C1 although they express similar level of the α5β1 integrin (Thomas *et al*, 2001a; 2001b). It is known that αv integrin subfamily (*e.g.* αvβ1, αvβ5) can bind to FN although with lower affinity than α5β1. The FN that is available for α5β1 may relatively decrease in the VB6 and C1 cell lines resulting in decreased internalization of *S. aureus* when compared to the H357 cells.

The results showing the rFnBPBD1-D4 protein and blocking antibodies for the integrin α5β1 had no effect on the internalization of *S. aureus* by NHK support

the observation that the interaction between NHK and *S. aureus* is FnBP-independent as previously discussed in Chapter 4.

Fibronectin is a key component of the provisional matrix during wound repair (Yamada and Clark, 1996b), and is often elevated during tissue remodeling and fibrosis and in neoplasia. *S. aureus* FnBPs act as a multivalent receptor and exhibit a high affinity for the 29 kDa amino-terminus of FN (Huff *et al*, 1994; Joh *et al*, 1998). It is considered that the *S. aureus* FnBPs can modulate cell behaviour upon binding to FN. Little is known about the biological activities of the *S. aureus* FnBPs on eukaryotic cells. A recent report has shown that the *S. aureus* FnBPs (rFnBPA proteins) can interact with human T lymphocytes via a FN bridge and the integrin $\alpha5\beta1$ to mediate adhesion and costimulatory signals (Miyamoto *et al*, 2001). In the present study, co-immobilization of rFnBPBD1-D4 with FN substrate inhibited haptotactic response of UP cells without alteration of cell adhesion ability. Addition of rFnBPBD1-D4 protein to wounded UP cell monolayers also inhibited directed migration with no effect on cell proliferation (BrdU incorporation) during 20 hours. The rFnBPBD1-D4 also inhibited polymerization of endogenous FN matrix on UP cell surface. FnBPs of *S. aureus* may therefore possess effects on epithelial cells distinct from mediation of bacterial internalization which are of biological implication in wound healing.

It has been shown that the $\alpha5\beta1$ integrin can transduce distinct signals upon interacting with either the amino-terminal or RGD region of FN (Hocking *et al*, 1998). Different regions of FN may serve to modulate intracellular signals during adhesion to FN and may be a mechanism by which cells regulate the rate and/or extent of migration. Adhesion of cells to r70 kDa amino-terminal FN fragment was shown to stimulate an increased haptotactic response compared to $rIII_{9,10}$ containing the RGD sequence (Hocking *et al*, 1998). In the present experiments, the ability of rFnBPBD1-D4 to inhibit both the haptotactic response and directed migration of cell monolayers suggests that the rFnBPBD1-D4 proteins compete for the binding of cells to the amino-terminal region of FN resulting in a reduced interaction with that region of FN and decreased cell migration. The inhibitory effect of the rFnBPBD1-D4 on cell migration was not accompanied by decreased proliferation of the cells.

This is in agreement with studies showing that an increase (Sarret *et al*, 1992; Atabai *et al*, 2002) or decrease (Kinsella *et al*, 2000) in cell migration rate is independent of cell proliferation.

Formation of a FN matrix is a cell-mediated process that involves initial interaction of the amino-terminus of FN with the cell surface and subsequent incorporation into a fibrillar ECM that is detergent insoluble (Magnusson and Mosher, 1998; Schwarzbauer and Sechler, 1999). The first five type I amino-terminal repeats of FN (I_{1-5}) is essential for FN matrix assembly. It binds to cells at matrix assembly sites and is the major domain for self-association of the FN fibrils (Aguirre *et al*, 1994). The rFnBPBD1-D4 proteins inhibited polymerization of endogenous FN by UP keratinocytes suggesting that the recombinant proteins may competitively block binding of the amino-terminus of FN to the cell surface and/or block FN self-assembly. It has been shown that the mAbs 9D2 and L8, which bind to the first type III module (III_1) of FN, inhibit FN polymerization into the ECM (Chernousov *et al*, 1991; Sottile *et al*, 2000; Sottile and Hocking, 2002). Despite its inhibitory activity in FN polymerization, the 9D2 antibody does not interfere with cell adhesion to FN (Chernousov *et al*, 1991). The present study also showed that the rFnBPBD1-D4 had no effect on the adhesion of keratinocytes to FN.

Although rFnBPBD1-D4 inhibited endogenous FN polymerization, it did not affect BrdU uptake of UP cells in wounded monolayers. This is in contrast to previous studies where FN effects on cell growth were linked to the presence or absence of a FN matrix (Bourdoulous *et al*, 1998; Sechler and Schwarzbauer, 1998; Sottile *et al*, 1998). The explanation by which growth inhibition was not observed in relation to the absence of endogenous FN matrix polymerization may be that the keratinocytes were already in a low proliferative state. The number of UP cells entering the cell cycle at 20 hours after wounding dropped about 7% (from 24% at 1 hour to 17.4% at 20 hours after injury). In addition, there may not be an absolute correlation between cell proliferation and FN assembly. For example, decorin expression enhanced FN fibrillogenesis by endothelial cells, but had no effect on cell proliferation (Kinsella *et al*, 2000). A recombinant fragment of FN (protein III_1-C) inhibited FN matrix assembly but did not affect the proliferation (^{3}H-thymidine

incorporation) of human endothelial cells (Mercurius and Morla, 1998). Moreover, a synthetic peptide from FN-binding protein F1 of *Streptococcus pyogenes* (FUD sequence) inhibited FN assembly, but did not affect stress fiber formation or growth (MTS assay) of human dermal fibroblasts or osteocarcoma cells (Tomasini-Johansson *et al*, 2001).

In summary, the present experiments show that *S. aureus* FnBPs and host cell integrin α5β1 were important in the internalization of *S. aureus* by immortalized keratinocytes. The internalization of *S. aureus* by primary keratinocytes was independent of *S. aureus* FnBPs and α5β1 integrin. A recombinant protein comprising D1-D4 repeats of *S. aureus* FnBPB possessed biological activities in that it inhibited the migration and endogenous FN polymerization, but did not affect the adhesion to FN or proliferation of UP keratinocytes.

Chapter 6

Effects of *Staphylococcus aureus* Culture Supernatants on Human Keratinocytes

6.1 Introduction

S. aureus is a common pathogen in cutaneous wound infections. Although *S. aureus* is commonly found in the nasal cavity and on skin, it can be isolated frequently from dental plaque and the oral cavity of healthy individuals (Smith *et al*, 2001). *S. aureus* produces a wide variety of exoproteins thought to be important in human diseases. Almost all *S. aureus* strains secrete a group of enzymes and cytotoxins, which includes nucleases, proteases, lipases, hyaluronidase, collagenase, and the four hemolysins (α, β, γ, and δ). Some of these enzymes and cytotoxins are capable of producing damage directly to the outer membrane of target cells. Some *S. aureus* strains produce additional exoproteins, which includes TSST-1, enterotoxins, exfoliative toxins and leukocidin. The TSST-1 and enterotoxins do not possess direct damaging action to host cells but can induce immune mediated damage through overproduction of cytokines derived from activated T cells and monocytes-macrophages (reviewed in Lowy, 1998).

Many of the *S. aureus* isolates from the oral cavity have been reported to be toxin-producing strains. Miyake *et al* (1991) reported 19% of *S. aureus* isolates from healthy children produced exfoliative toxin and 40% produced enterotoxin. In addition, TSST-1-producing strains have been isolated from staphylococcal mucositis (Bagg *et al*, 1995). A study of 94 *S. aureus* isolates from recurrent skin and respiratory tract infections demonstrated that 79% of the isolates produced α-toxin exclusively or in combination with δ- or β-toxin. Five percent of *S. aureus* isolates from the respiratory tract produced β-toxin whereas 25% from the skin infections (Slobodnikova *et al*, 1995). This suggests that heavy colonization of the oral cavity by *S. aureus* may lead to local mucosal damage.

The production of secreted exoproteins and surface proteins of *S. aureus* is coordinately regulated in a growth phase-dependent manner. Staphylococcal surface proteins such as staphylococcal protein A and the fibronectin-binding proteins

(FnBPs) are expressed maximally by exponentially growing cells (Proctor *et al*, 1982; Saravia-Otten *et al*, 1997). The synthesis of exoproteins such as hemolysins, proteases, and TSST-1 takes place mainly during the post-exponential phase, when synthesis of the surface proteins has ceased. It has been demonstrated that the cell wall-associated FnBPs and protein A are actively released from the bacterial surface soon after synthesis has been turned off through the activity of a staphylococcal serine protease (Karlsson *et al*, 2001). Accordingly, both exponential (containing no or little toxin, and presumably few fragments of released surface proteins) and stationary (containing high level of toxins and other exoproteins/enzymes) phase growth media from *S. aureus* were included in the study.

Since these secreted products may contribute to delayed healing or mucosal damage, the aim of this study was to examine the effects of culture supernatants from *S. aureus* on the proliferation, adhesion and migration of human keratinocytes.

6.2 Materials and Methods

6.2.1 Keratinocytes

Human keratinocytes used in the study were the immortalized UP cell line and primary oral keratinocytes (NHK). The keratinocytes were maintained in KGM as described in Section 2.2.

6.2.2 Preparation of Bacterial Culture Supernatants

Three strains of *S. aureus* were included in the study: NCTC6571, 8325-4 and LS-1. Strain NCTC6571 is a laboratory strain commonly used as a control in an antibiotic sensitivity test. Strain 8325-4 is a laboratory strain commonly used for genetic studies and has been reported to remain virulent in animal models of infection (Bramley *et al*, 1989). Strain LS-1 is a pathogenic strain isolated from a mouse with septic arthritis (Bremell *et al*, 1990; 1992). Culture supernatants of both exponential (three and half hours) and stationary (18 hours) growth phases from *S. aureus* strains NCTC6571, 8325-4, and LS-1 were prepared as described in Section 2.11.

6.2.3 Phase Contrast Microscopy

To observe changes in cell morphology after exposure to the bacterial culture supernatants, keratinocytes were plated at a concentration of 70,000 cells per well in 24-well plates. After an overnight incubation, cells were washed and exposed to 5%, 25%, or 50% concentration of *S. aureus* culture supernatant diluted in DMEM. Cells in control wells were incubated with dilutions of THB. Cell morphology changes were assessed visually after 24 h with an inverted microscope (model Leica DM IRB) equipped with a Leica DC200 digital camera (Leica Microsystems Wetzlar GmbH, Germany).

6.2.4 Cell Proliferation Assay

Cell proliferation assays were performed in 96-well plates as described in Section 2.4. Cells were incubated with 5%, 25%, or 50% *S. aureus* culture supernatant diluted in DMEM. After incubation for 6 hours (acute exposure) or 24 hours (chronic exposure) at 37°C, cells were washed twice by immersing the plate gently in PBS. The relative number of adherent viable cells in each well was quantified by the MTS assay as described in Section 2.3. Cells in control wells were incubated with dilutions of THB.

6.2.5 Keratinocyte Adhesion and Migration on Fibronectin

A 25% *S. aureus* culture supernatant was used to determine the effect of the bacterial products on keratinocyte adhesion to and migration on FN-coated surfaces using the methods described in Sections 2.5 and 2.6 respectively. Prior to the assessment of adhesion and migration, the viability of keratinocytes in suspension following incubation with the 25% culture supernatants from strain 8325-4 was confirmed by staining with 0.2% trypan blue (1:1 v/v; Sigma®). Viability was estimated by counting approximately 200 cells using the hemocytometer, and calculating the percentage of cells excluding the stain. Suspensions of UP cells and NHK were incubated in the presence of supernatants for 30 minutes at room temperature prior to plating for adhesion or migration assays. Cells incubated with the same dilutions of sterile THB served as controls. Cells, in the presence of the

bacterial culture supernatants or THB, were allowed to adhere for 2 hours or migrate for 4 hours.

6.2.6 Flow Cytometric Analysis of Surface Integrin Expression on Keratinocytes Treated with 25% Culture Supernatants from Strain 8325-4

NHK and UP keratinocytes grown to subconfluence in 80-cm^2 tissue culture flasks were incubated with 25% exponential or stationary phase culture supernatant from *S. aureus* strain 8325-4 diluted in KGM. Control flasks were incubated in KGM alone. After 18 hours, cells were washed and harvested for indirect immuno-fluorescence using antibodies against α5 (P1D6), β1 (TDM29), αv (L230), and β6 (R6G9) integrin subunits as described in Section 2.8. Sources and dilutions of the antibodies are listed in Table 2-1. Surface expression of the integrins was analyzed by flow cytometry.

6.2.7 Treatment of Bacterial Culture Supernatants with Heat or Protease Inhibitor

To investigate the effect of heat and protease inhibitor on the activity of bacterial culture supernatants on keratinocyte migration, stationary phase culture supernatants from strains 8325-4 and LS-1 were heated in a water-bath at 56°C or incubated with 50 μg/ml α_2-macroglobulin (α_2M) protease inhibitor (Sigma®) at 37°C for 30 min before use.

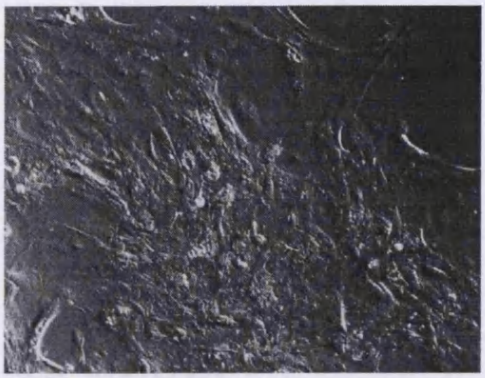
6.2.8 Hemolytic Activity Assay

Hemolytic activity against rabbit or sheep erythrocytes is a common method of determining the presumptive presence of α- or β-toxin respectively. The presence of α- and β-toxin in the culture supernatants from *S. aureus* strains NCTC6571, 8325-4, and LS-1 was determined as described in Section 2.12.

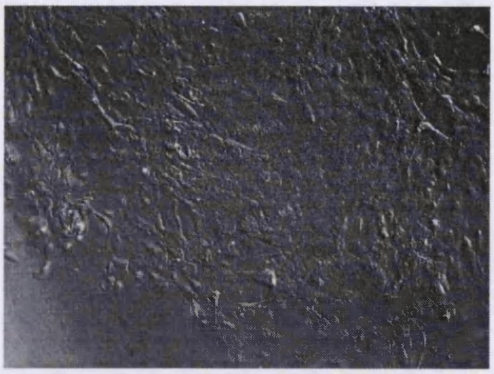
6.3 Results

6.3.1 Morphological Studies

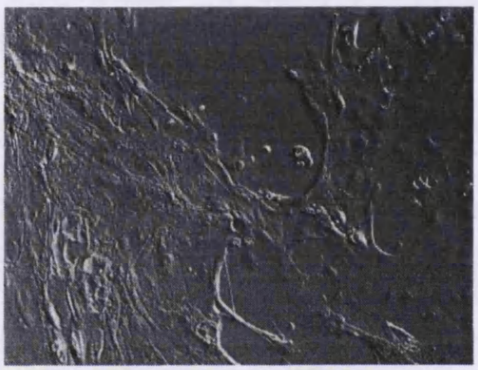
Cell detachment and morphology of NHK after exposure to *S. aureus* exponential phase culture supernatants from all three strains tested were similar to the control cultures (Figure 6-1). Exposure to *S. aureus* stationary phase culture supernatants resulted in strain- and concentration-dependent change in morphology (Figure 6-2). NHK exhibited morphologic rounding and shrinking, and increased cell detachment when exposed to stationary phase culture supernatants which was most obvious with the 50% concentration, and with strain 8325-4. Cell detachment and morphology of NHK after exposure to stationary phase culture supernatants from strain NCTC6571 were similar to the control cultures. Similar cell morphology changes and detachment were obtained with UP keratinocytes (data not shown).



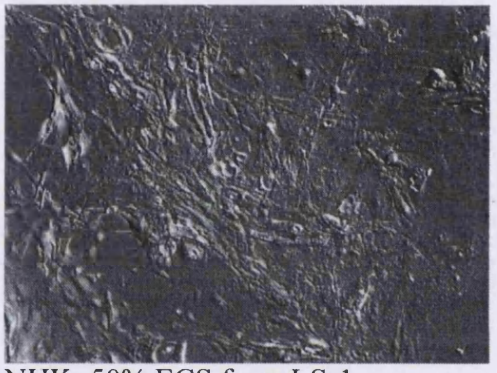
NHK: 50% THB



NHK: 50% ECS from NCTC6571

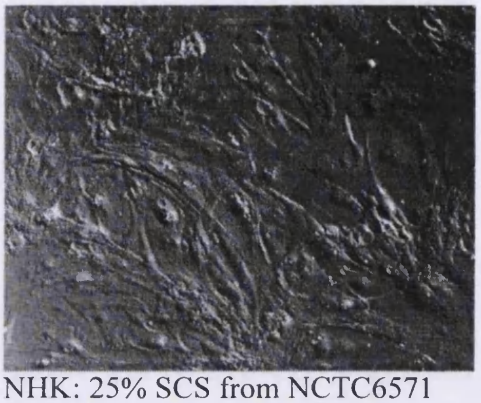


NHK: 50% ECS from 8325-4

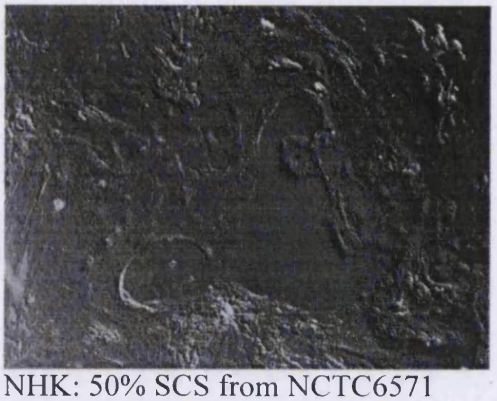


NHK: 50% ECS from LS-1

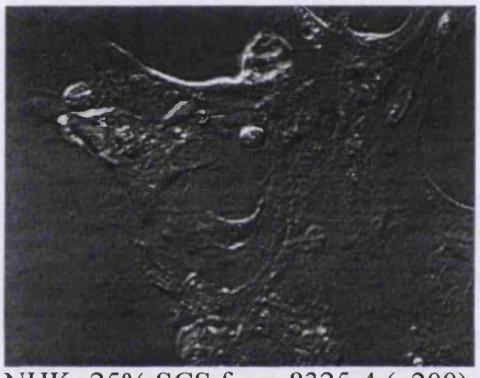
Figure 6-1 Phase contrast micrographs of primary oral keratinocytes (NHK) after exposure to 50% *S. aureus* exponential phase culture supernatants (ECS) for 24 hours. Control cultures were incubated in 50% Todd Hewitt broth (THB). The pictures were taken after rinsing the cultures once with PBS (original magnification: x100).



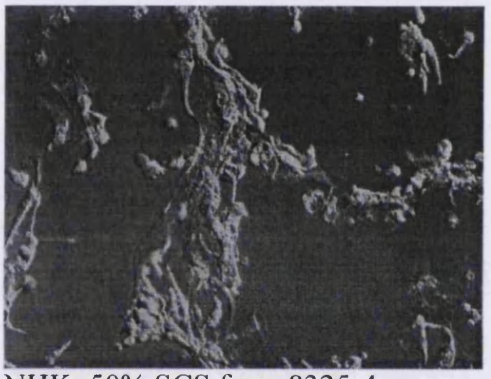
NHK: 25% SCS from NCTC6571



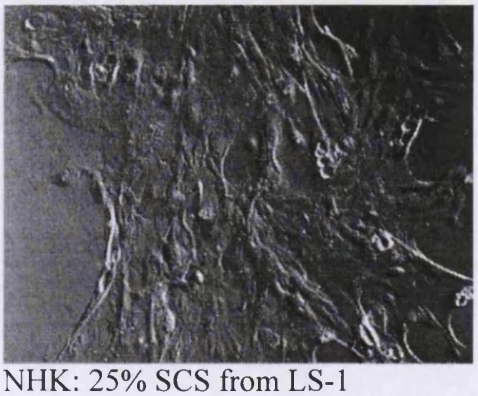
NHK: 50% SCS from NCTC6571



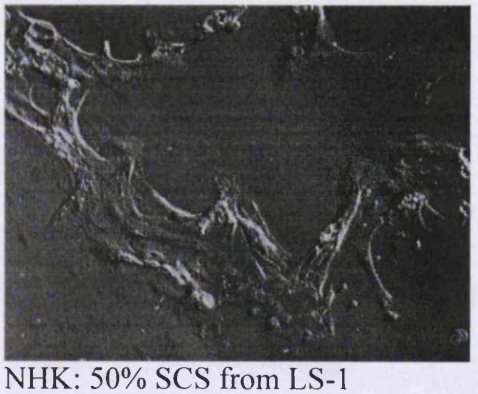
NHK: 25% SCS from 8325-4 (x200)



NHK: 50% SCS from 8325-4



NHK: 25% SCS from LS-1



NHK: 50% SCS from LS-1

Figure 6-2 Phase contrast micrographs of primary oral keratinocytes (NHK) after exposure to 25% (left column) or 50% (right column) *S. aureus* stationary phase culture supernatants (SCS) for 24 hours. The pictures were taken after rinsing the cultures once with PBS (original magnification: x100 unless specified).

6.3.2 Effects of Culture Supernatants from *S. aureus* Strain NCTC6571 on Keratinocytes

6.3.2.1 Effect on Cell Proliferation

The culture supernatants from both exponential and stationary growth phases of *S. aureus* strain NCTC6571 had no effect on the proliferation of either UP cells or NHK (Table 6-1). The growth rate of both UP cells and NHK was not significantly different from the control cultures after culturing for 24 hours. In contrast, the culture supernatants at 25% and 50% concentration exhibited a tendency to stimulate low-level proliferative effect on keratinocytes during short-term exposure of 6 hours, but this was not statistically significant using one sample t-test.

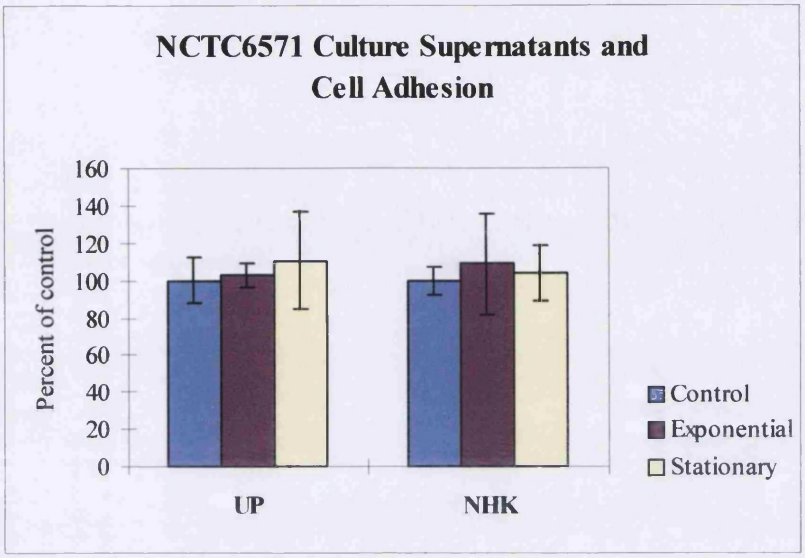
6.3.2.2 Effect on Cell Adhesion and Migration

The effect of culture supernatants from strain NCTC6571 on the adhesion to and migration on fibronectin of both UP cells and NHK is shown in Figure 6-3. Exposure to 25% culture supernatants of both exponential and stationary phases had no effect on cell adhesion and migration compared with the adhesion and migration in control wells.

Table 6-1 Effect of culture supernatants (CS) from *S. aureus* strain NCTC6571 on keratinocyte proliferation.

	MTS (% of control)		
	5%CS	25%CS	50%CS
UP:			
Exponential			
6 h	95.46 ± 20.16	113.02 ± 18.95	112.02 ± 47.49
24 h	101.91 ± 21.63	84.92 ± 12.95	95.58 ± 16.29
Stationary			
6 h	88.83 ± 5.66	121.33 ± 24.73	107.95 ± 6.23
24 h	89.46 ± 26.08	99.59 ± 38.55	93.15 ± 11.41
NHK:			
Exponential			
6 h	114.25 ± 27.85	106.07 ± 8.12	118.30 ± 8.04
24 h	84.04 ± 22.03	113.54 ± 23.56	116.80 ± 47.36
Stationary			
6 h	95.85 ± 18.69	144.17 ± 64.25	137.45 ± 40.56
24 h	92.22 ± 52.34	102.21 ± 35.93	110.02 ± 48.54

Relative cell number was determined by the MTS assay. Control cells were treated with the same dilutions of Todd Hewitt broth and represented the 100% values in each experiment. The values represent mean ± standard deviation of three independent experiments performed in triplicate. No statistical significance ($P > 0.05$) was observed using one sample t-test compared with 100% value of the controls.



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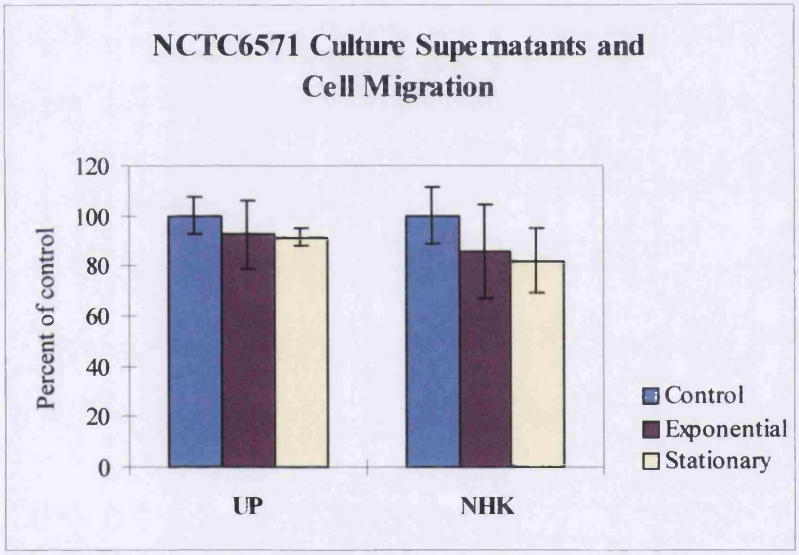


(b)

Figure 6-3 Effects of 25% culture supernatants from *S. aureus* strain NCTC6571 on the adhesion (a) and migration (b) of human keratinocytes (NHK and UP) on fibronectin-coated surfaces. Control cells were treated with 25% concentration of Todd Hewitt broth and represented the 100% values in each experiment. The values represent mean ± standard deviation of three independent experiments performed in triplicate.

6.3.3 Effect of Culture Supernatants from *S. aureus* Strain 8325-4 on Keratinocytes

6.3.3.1 Effect on Cell Proliferation

The effect of culture supernatants from *S. aureus* strain 8325-4 on the proliferation of UP keratinocyte and NHK was growth phase dependent (Table 6-2). The exponential phase culture supernatant had no significant inhibitory effects on keratinocyte proliferation at 6 or 24 hours. The stationary phase culture supernatant inhibited cell proliferation as determined by the MTS assay in a dose- and time-dependent manner. The decreased relative cell number of NHK compared to control cultures after exposure to the 50% stationary phase culture supernatants for 24 hours correlated with the degree of cell detachment observed under phase contrast microscopy described previously. However, short-term exposure for 6 hours to the 25% culture supernatant had no significant inhibitory effects on keratinocyte proliferation compared to control cultures. Thus, the 25% concentration was used in the following experiments.

Table 6-2 Effect of culture supernatants (CS) from *S. aureus* strain 8325-4 on keratinocyte proliferation.

	MTS (% of control)		
	5%CS	25%CS	50%CS
UP:			
Exponential			
6 h	87.38 ± 20.25	104.75 ± 10.87	89.90 ± 42.25
24 h	103.97 ± 22.29	76.40 ± 17.17	87.28 ± 26.38
Stationary			
6 h	104.75 ± 46.14	82.67 ± 37.50	36.22 ± 12.58 *(p=0.02)*
24 h	86.77 ± 11.59	56.64 ± 15.21 *(p=0.01)*	12.13 ± 1.94 *(p=0.0002)*
NHK:			
Exponential			
6 h	115.29 ± 15.50	88.45 ± 21.33	110.12 ± 8.13
24 h	98.79 ± 18.79	86.31 ± 12.63	95.50 ± 6.21
Stationary			
6 h	113.87 ± 46.26	92.95 ± 44.39	71.95 ± 8.33 *(p=0.03)*
24 h	103.42 ± 36.56	48.97 ± 11.74 *(p=0.0001)*	28.99 ± 13.93 *(p=0.01)*

Relative cell number was determined by the MTS assay. Control cells were treated with the same dilutions of Todd Hewitt broth and represented the 100% values in each experiment. The values represent mean ± standard deviation of at least three independent experiments performed in triplicate. Statistical significance was performed using one sample t-test compared with 100% value of the controls.

6.3.3.2 Effect on Cell Adhesion and Migration

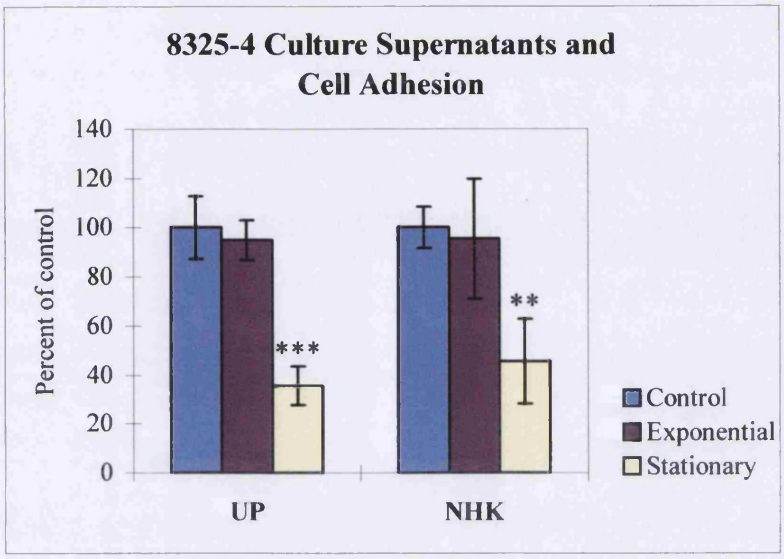
The viability of treated-keratinocytes was not different from the untreated controls in either adhesion or migration medium (Table 6-3). This confirms that the concentration used (25%) had no lethal effect on cells in suspension during the assays.

Table 6-3 Viability of keratinocytes in suspension after exposure to 25% 8325-4 exponential (E) or stationary (S) phase culture supernatant (CS) diluted in migration buffer (MB) or serum free medium (SFM) before the migration and adhesion assays respectively.

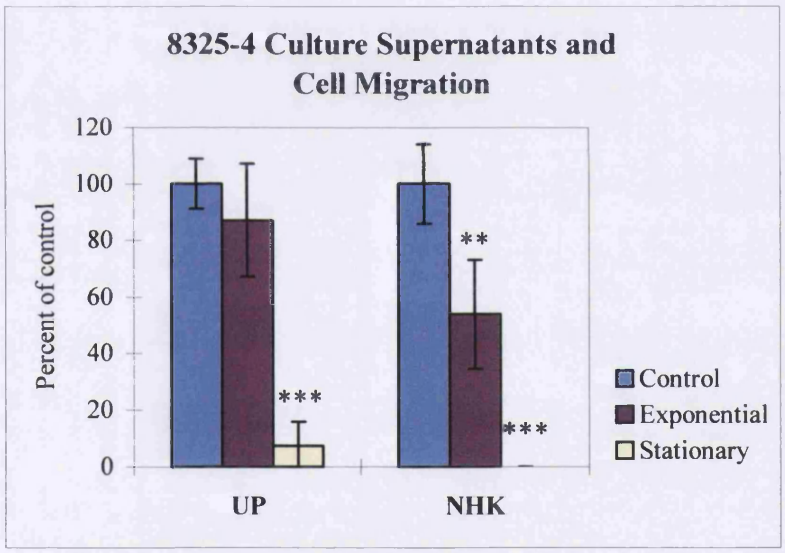
	Trypan Blue Exclusion Test (% viable cells)		
	Todd Hewitt broth	8325-4ECS	8325-4SCS
NHK :			
in MB (n=4)	95.42 ± 2.05	94.77 ± 0.85	93.07 ± 2.19
in SFM (n=4)	89.39 ± 9.68	91.75 ± 5.39	81.11 ± 13.69
UP:			
in MB (n=6)	96.60 ± 0.62	96.48 ± 1.04	95.59 ± 0.67
in SFM (n=3)	92.74 ± 0.89	91.51 ± 2.14	91.83 ± 1.43

No statistical significance ($p > 0.05$) was observed when compared to the controls treated with Todd Hewitt broth.

Figure 6-4 shows keratinocyte adhesion and migration in the presence of 25% 8325-4 culture supernatants. Compared to control wells in 25% THB, the exponential phase culture supernatant did not affect cell adhesion but inhibited the migration of NHK by approximately 45% ($p = 0.006$). In contrast, the stationary phase culture supernatant significantly inhibited both cell adhesion and migration. The adhesion of keratinocytes on fibronectin was reduced to approximately 40%-50% of the controls, and the migration was almost totally abolished.



(a)



(b)

Figure 6-4 Effects of 25% culture supernatants from *S. aureus* strain 8325-4 on the adhesion (a) and migration (b) of human keratinocytes (NHK and UP) on fibronectin-coated surfaces. Control cells were treated with 25% Todd Hewitt broth and represented the 100% values in each experiment. The values represent mean ± standard deviation of four to five independent experiments performed in triplicate. Statistical significance was performed using one sample t-test compared with 100% value of the controls. **, $p < 0.01$; ***, $p < 0.001$.

6.3.4 Effect of Culture Supernatants from *S. aureus* Strain LS-1 on Keratinocytes

6.3.4.1 Effect on Cell Proliferation

The effect of culture supernatants from *S. aureus* strain LS-1 on keratinocyte proliferation was highly variable, particularly with NHK (Table 6-4). The exponential phase culture supernatant exhibited a tendency to stimulate low-level proliferation of UP keratinocyte. However, the stationary phase culture supernatant inhibited cell proliferation at 24 hours.

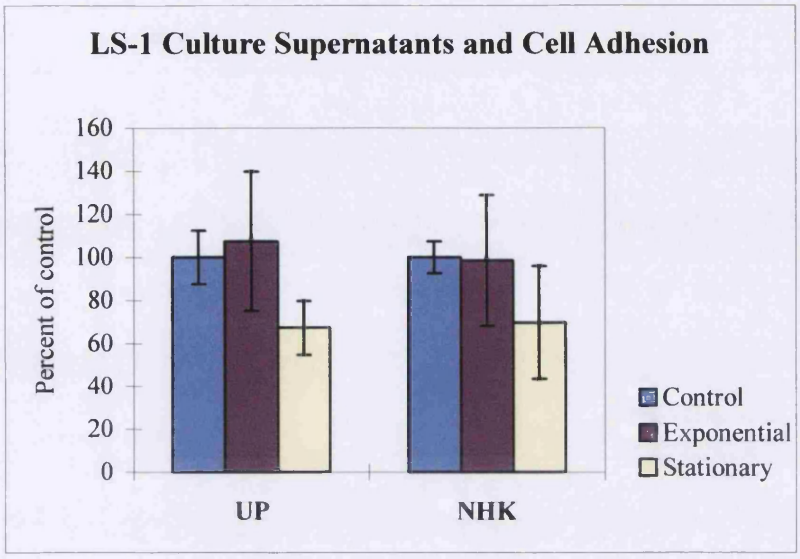
6.3.4.2 Effect on Cell Adhesion and Migration

The effect of 25% culture supernatants from strain LS-1 on the adhesion to and migration on fibronectin of UP keratinocyte and NHK is shown in Figure 6-5. Exposure to the exponential phase culture supernatant from LS-1 had no effect on the adhesion and migration of UP or NHK compared with controls. The stationary phase culture supernatant from LS-1 reduced keratinocyte adhesion by approximately 30%, and markedly inhibited the migration by 80%-90%.

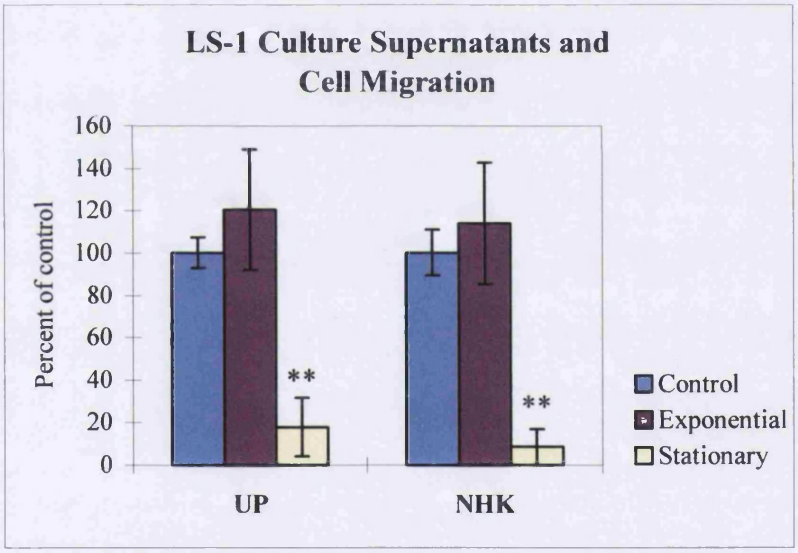
Table 6-4 Effect of culture supernatants (CS) from *S. aureus* strain LS-1 on keratinocyte proliferation.

	MTS (% of control)		
	5%CS	25%CS	50%CS
UP:			
Exponential			
6 h	117.10 ± 30.53	129.94 ± 14.41 *(p=0.004)*	144.77 ± 18.86
24 h	102.40 ± 29.67	94.73 ± 14.04	107.20 ± 37.12
Stationary			
6 h	87.17 ± 25.08	146.41 ± 38.55	105.70 ± 10.63
24 h	73.69 ± 15.88 *(p=0.055)*	76.56 ± 15.10 *(p=0.053)*	86.91 ± 21.15
NHK:			
Exponential			
6 h	96.70 ± 15.68	77.13 ± 4.22 *(p=0.01)*	86.53 ± 40.28
24 h	61.54 ± 31.26	81.13 ± 47.25	79.52 ± 53.48
Stationary			
6 h	75.11 ± 23.58	98.80 ± 37.44	120.39 ± 22.84
24 h	58.33 ± 31.24	69.72 ± 41.25	70.68 ± 59.35

Relative cell number was determined by the MTS assay. Control cells were treated with the same dilutions of Todd Hewitt broth and represented the 100% values in each experiment. The values represent mean ± standard deviation of three independent experiments performed in triplicate. Statistical significance was performed using one sample t-test compared with 100% value of the controls.



(a)



(b)

Figure 6-5 Effects of 25% culture supernatants from *S. aureus* strain LS-1 on the adhesion (a) and migration (b) of human keratinocytes (NHK and UP) on fibronectin-coated surfaces. Control cells were treated with 25% Todd Hewitt broth and represented the 100% value in each experiment. The values represent mean ± standard deviation of three independent experiments performed in triplicate. Statistical significance was performed using one sample t-test compared with 100% value of the controls. **, $p < 0.01$.

6.3.5 Surface Expression of Fibronectin-binding Integrins on Keratinocytes after Exposure to Exponential and Stationary Phase Culture Supernatants from *S. aureus* Strain 8325-4

The presence of staphylococcal proteases in the stationary phase culture supernatant may degrade fibronectin substrate or damage the fibronectin receptors on keratinocytes, which in turn may affect cell adhesion and migration. To elucidate the decreased adhesion and migration of UP and NHK on fibronectin after treatment with 8325-4 culture supernatants, surface expression of fibronectin-binding integrins, $\alpha5\beta1$ and $\alpha v\beta6$, on keratinocytes was analyzed by flow cytometry. Keratinocytes in KGM alone were used as the control. Only the adherent cells were harvested and analyzed for surface integrin expression. Cell detachment and morphology of keratinocytes treated with the exponential phase culture supernatant were similar to the control cultures. The number of attached cells collected from the stationary phase culture supernatant-treated flasks were approximately 57% for UP and 35% for NHK. Cell vitality of the culture supernatant-treated UP (>90%) was similar to the untreated control cultures. Percentage of viable cells of the attached stationary phase culture supernatant-treated NHK was approximately 80% compared to 90% of the untreated controls as determined by trypan blue exclusion test and propidium iodide staining prior to flow cytometric analysis (see Appendix C).

Histograms of the surface integrin expression on NHK and UP are shown in Figures 6-6 and 6-7 respectively. The expression profiles of $\alpha5$ (P1D6), $\beta1$ (TDM29), αv (L230) and $\beta6$ (R6G9) integrin subunits on NHK did not change after 18 hours of exposure to either exponential or stationary phase culture supernatant of 8325-4. Similar results were also obtained with 4 hours exposure (data not shown). A slight decrease in the integrin expression on UP cells following exposure to the stationary phase culture supernatant was observed, but this was not consistent. These results suggest that decreased cell adhesion and migration caused by staphylococcal secreted products does not involve the loss of cell surface integrin expression.

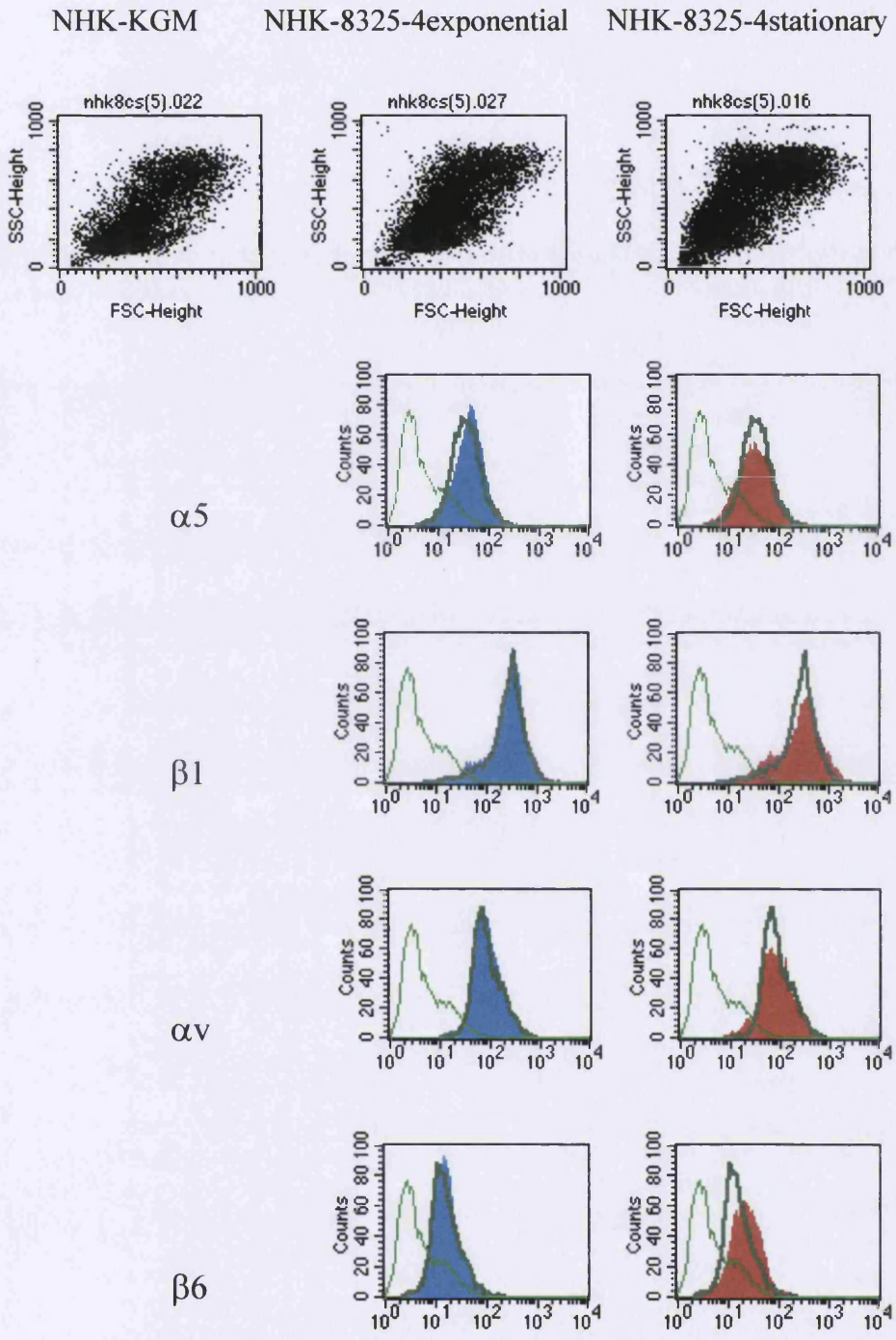


Figure 6-6 Flow cytometric analysis of NHK after exposure to 25% *S. aureus* 8325-4 culture supernatants (filled peaks) for 18 hours. Cells incubated in KGM alone were used as control (thick line peak). NHK were immunolabeled with antibodies to α5 (P1D6), β1 (TDM29), αv (L230) or β6 (R6G9) integrin. Negative control (thin line peak) had secondary antibody only. The dot plots show forward versus side scatter distribution of untreated, exponential supernatant-treated, and stationary supernatant-treated NHK.

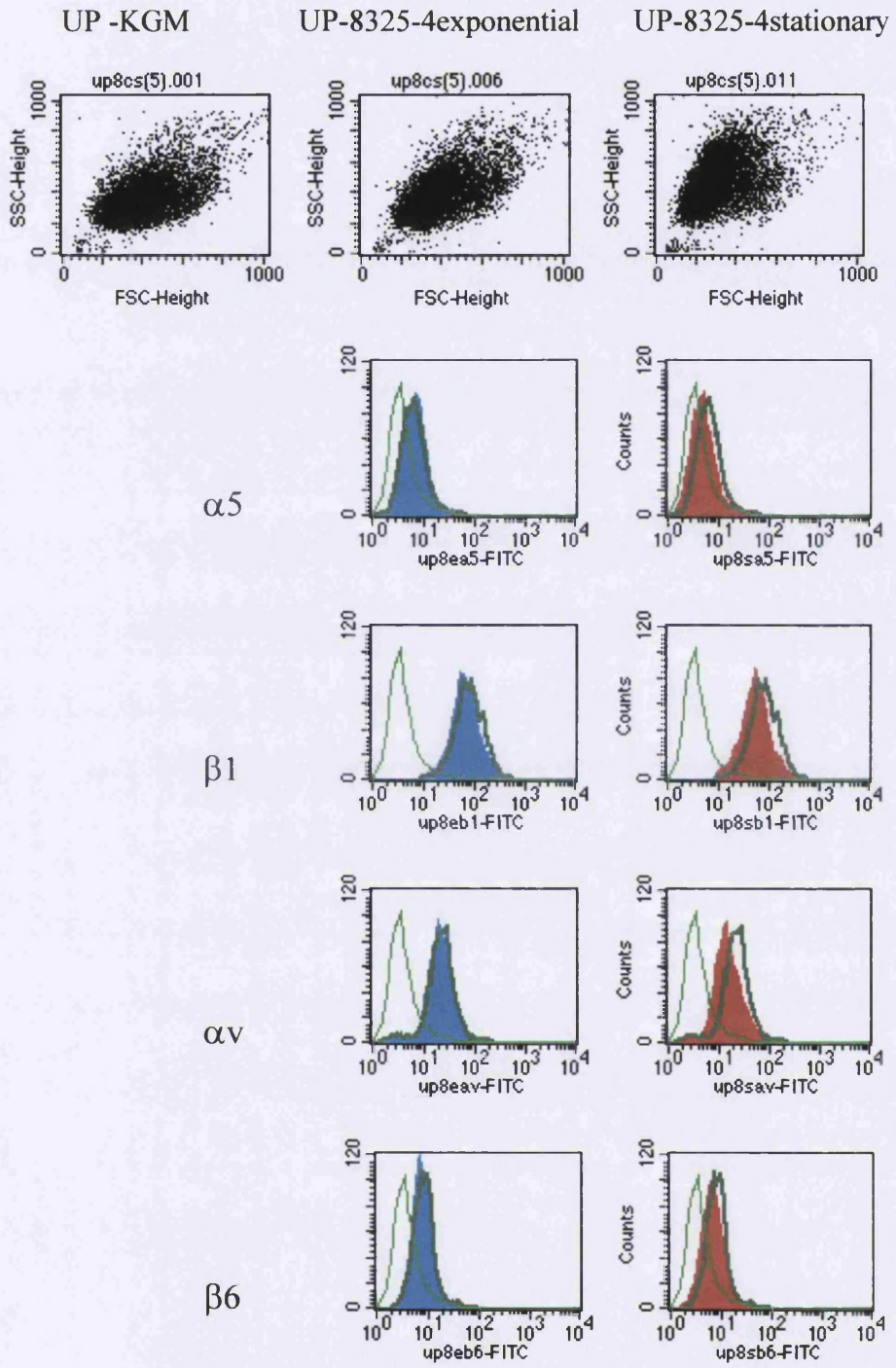
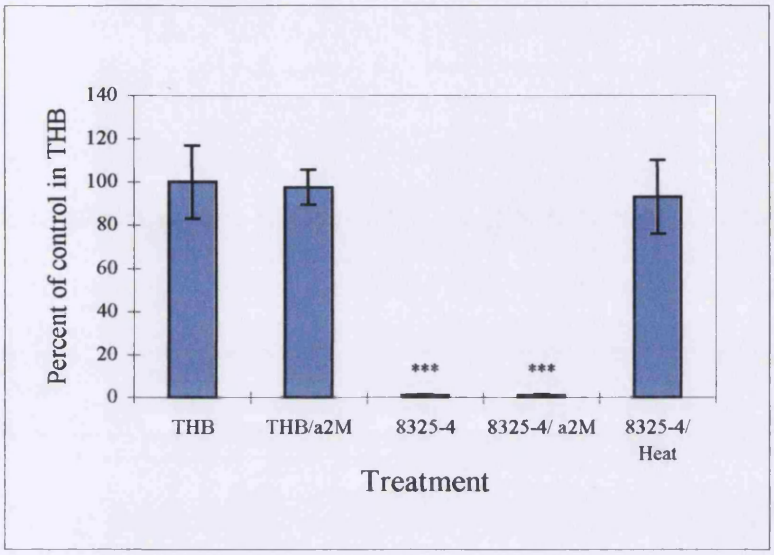


Figure 6-7 Flow cytometric analysis of UP cells after exposure to 25% *S. aureus* 8325-4 culture supernatants (filled peak) for 18 hours. Cells incubated in KGM alone were used as control (thick line peak). UP cells were immunolabeled with antibodies to α5 (P1D6), β1 (TDM29), αv (L230) or β6 (R6G9) integrin. Negative control (thin line peak) had secondary antibody only. The dot plots show forward versus side scatter distribution of untreated, exponential supernatant-treated, and stationary supernatant-treated UP.

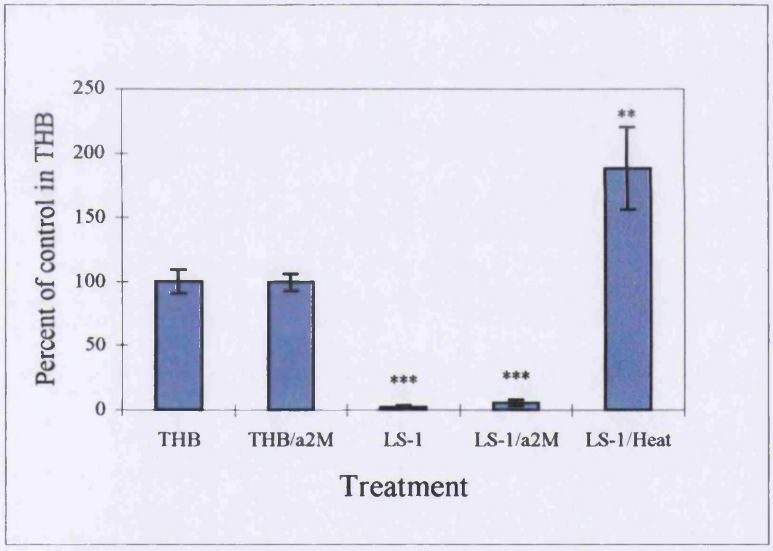
6.3.6 Characterization of the Inhibitory Factor of *S. aureus*

Treatment of the stationary phase culture supernatants from both *S. aureus* strains 8325-4 and LS-1 with heat at 56°C for 30 min eliminated its inhibitory function on the migration of NHK (Figure 6-8) and UP (Figure 6-9). However, treatment with a broad spectrum protease inhibitor, α_2-macroglobulin, did not eliminate its ability to inhibit cell migration. The results suggest that the inhibitory effects on cell adhesion and migration are unlikely to be mediated by a protease.

Studies of 8325-4 grown in brain heart infusion (BHI) medium have shown that the most prominent secreted protein in the 8325-4 profile was α-hemolysin (Chan and Foster, 1998a). The most prominent proteins in the LS-1 profile were TSST-1 and β-hemolysin (Akingbadé *et al*, 2000). Accordingly, hemolytic activity in the *S. aureus* culture supernatant was measured and the results are shown in Figure 6-10. The stationary phase culture supernatants from strains 8325-4 and LS-1, but not NCTC6571, were able to hemolyse rabbit (α-toxin) and sheep (β-toxin) erythrocytes. The hemolytic titers of α- and β-toxin for 8325-4 were 32 and 4 respectively. The LS-1 stationary phase culture supernatant contained slightly less α-toxin hemolytic activity than the 8325-4, but it exhibited the highest β-toxin hemolytic activity with hemolytic titer of 16. As exoproteins and toxins are mainly produced during post-exponential phase and stationary phase, all exponential phase culture supernatants from *S. aureus* strains 8325-4, LS-1 and NCTC6571 in the present study contained no detectable α- or β-toxin.

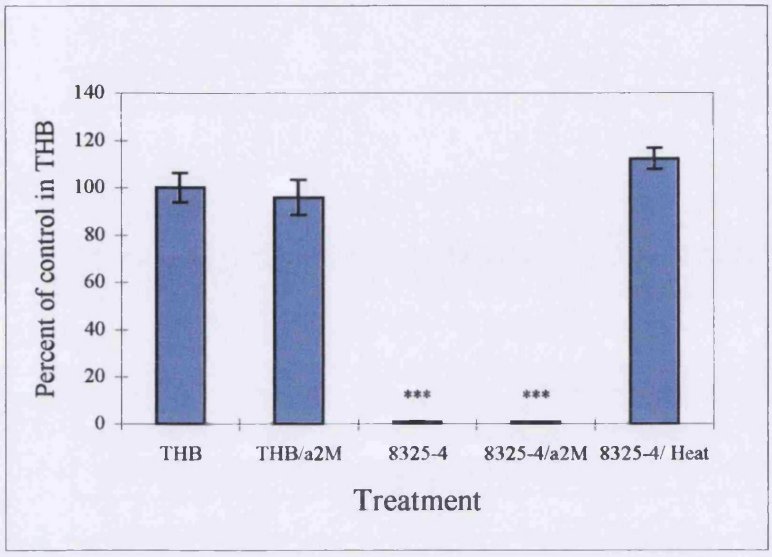


(a)



(b)

Figure 6-8 The migration of NHK in the presence of 25% 8325-4 (a) or LS-1 (b) stationary phase culture supernatant prior to and after treatment with heating or α_2-macroglobulin protease inhibitor (a2M). Control cells were incubated with 25% Todd Hewitt broth (THB) alone or with 25% THB plus a2M. Data are expressed as mean of percent of control in THB ± standard deviation from a representative experiment performed in triplicate. The experiments were repeated twice and showed similar results. Significantly different from the control in THB, **, $p = 0.01$; ***, $p < 0.001$.



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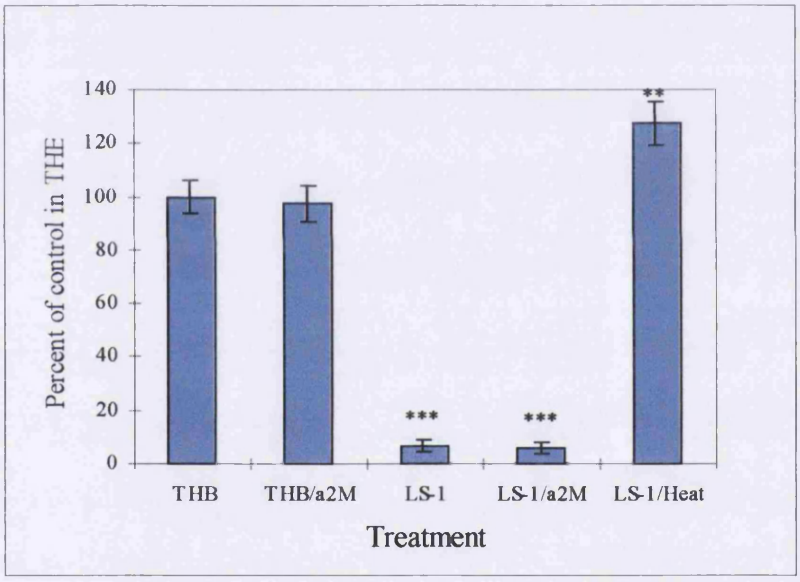
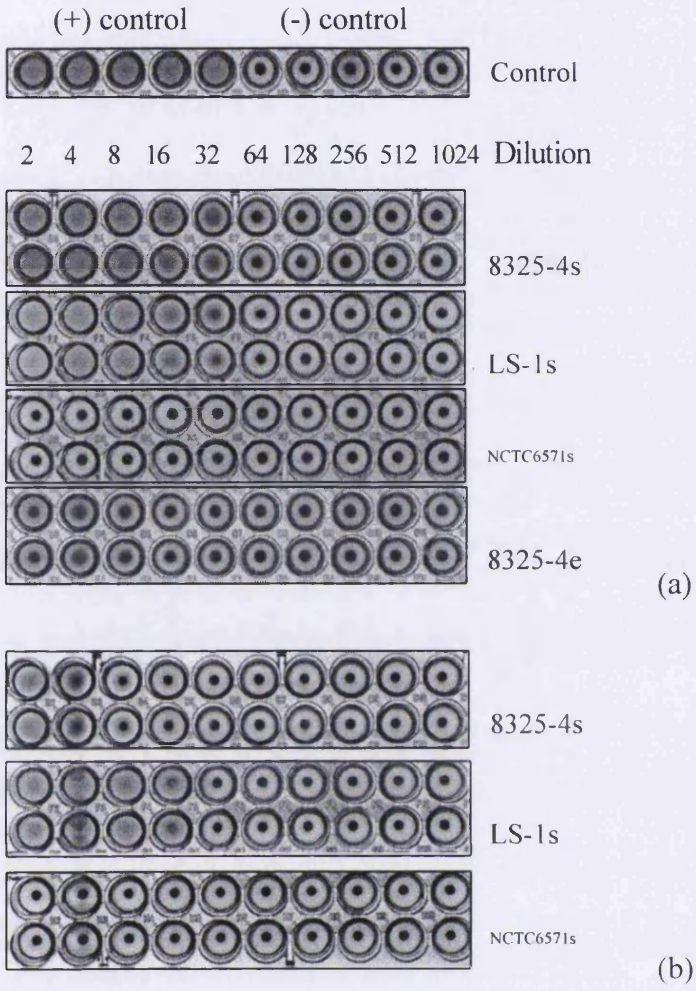


(b)

Figure 6-9 The migration of UP keratinocytes in the presence of 25% 8325-4 (a) or LS-1 (b) stationary phase culture supernatant prior to and after treatment with heating or α_2-macroglobulin protease inhibitor (a2M). Control cells were incubated with 25% Todd Hewitt broth (THB) alone or with 25% THB plus a2M. Data are expressed as mean of percent of control in THB ± standard deviation from a representative experiment performed in triplicate. The experiments were repeated twice and showed similar results. Significantly different from the control in THB, **, $p = 0.01$; ***, $p < 0.001$.



S. aureus strain	Hemolytic titer	
	α-toxin	β-toxin
8325-4	32	4
LS-1	16-32	16
NCTC6571	0	0

Figure 6-10 *In vitro* hemolysin production by *S. aureus*. Each stationary phase culture supernatant was titered for hemolytic activity against rabbit (a) or sheep (b) erythrocytes. The hemolytic titer is the highest dilution giving rise to lysis of rabbit (α-toxin) or sheep (β-toxin) erythrocytes. Control wells were incubated with water (positive control, five wells on the left) or PBS (negative control, five wells on the right). The hemolytic activity of 8325-4 exponential phase culture supernatant (8325-4e) against rabbit erythrocytes is shown.

6.4 Discussion

In this study, the effects of *S. aureus* culture supernatants on the proliferation, adhesion and migration of human keratinocytes (UP and NHK) were tested *in vitro*. Three *S. aureus* strains were included in the study. *S. aureus* is a pathogen, which escapes the host defenses and damages tissue by producing numerous virulence factors. These virulence factors include surface proteins, such as fibronectin-binding proteins, protein A, and a number of exoenzymes. *S. aureus* also produces several toxins including exfoliative toxins, enterotoxins, leucocidins, TSST-1, and cytotoxins (α-, β-, γ-, and δ-toxin).

It was considered that the laboratory strain NCTC6571, which was internalized at a very low level by keratinocytes, might possess less virulence than strain LS-1 which was highly internalized and is virulent in animal models. Indeed, strain NCTC6571, in which cytotoxins could not be detected in the culture supernatants, did not show an inhibitory effect on keratinocyte proliferation. On the contrary, it exhibited a tendency to stimulate low-level proliferation of keratinocytes during acute or short-term exposure of 6 hours. In the absence of cytotoxins, other released surface elements and proteins such as peptidoglycan, protein A and lipoteichoic acid (LTA) may affect the keratinocytes. LTA from *S. aureus* has been reported to activate the platelet-activating factor resulting in the activation of EGF-receptor in mucosal epithelial cells (Lemjabbar and Basbaum, 2002). The activation of EGF-receptor could lead to keratinocyte survival and proliferation (Jost *et al*, 2001). The secreted products from NCTC6571 at 25% concentration did not modulate adhesion or migration responses in keratinocytes.

The effect of culture supernatants from *S. aureus* strain 8325-4 on keratinocyte proliferation, adhesion and migration, however, was growth phase dependent. The stationary phase culture supernatant exhibited inhibitory effects on keratinocyte proliferation in a dose- and time-dependent manner. It also inhibited cell adhesion and migration. Although *S. aureus* strain 8325-4 is a laboratory strain cured of prophages, it remains virulent in animal models of infection. *S. aureus* strain 8325-4 caused acute mastitis and death within 48 h for 60% of the mice inoculated (Bramley *et al*, 1989). In a murine arthritis model, the majority of mice injected with

8325-4 developed arthritis and almost half displayed an erosive arthropathy (Abdelnour *et al*, 1993). *S. aureus* strain LS-1 is commonly used in animal models of septic arthritis (Bremell *et al*, 1991; 1992; Hultgren *et al*, 1998; Sakiniene *et al*, 1999) because of its highly virulent character. However, its inhibitory effect on the proliferation of human keratinocytes was less prominent than that of 8325-4. The differences in the response of cell proliferation and morphological changes to different *S. aureus* strains are likely to be due to differences in bacterial protein production by these strains. An *in vitro* study of freezed-dried supernatants prepared from clinical isolates of *S. aureus* from burn lesions has demonstrated variable strain-dependent results with regard to their ability to cause detachment and death of both human keratinocytes and fibroblasts (Taylor *et al*, 1990).

Cell migration is important in many processes, including embryonic development, wound healing and the immune response. The migratory response of NHK and UP cells during exposure to 25% *S. aureus* culture supernatants was determined. The 25% dilution was chosen because it had no lethal effect on keratinocytes in suspension during the assay. The present results show that stationary phase culture supernatants from *S. aureus* strains 8325-4 and LS-1 completely inhibited cell migration on fibronectin. This migration inhibition effect was associated with reduced cell adhesion ability, particularly with the stationary phase culture supernatant from 8325-4. The exponential phase bacterial supernatants had no effect on cell migration and adhesion on fibronectin. However, the exponential phase culture supernatant from strain 8325-4 exhibited approximately 40% migration inhibition on primary keratinocyte (NHK) but not immortalized keratinocyte (UP), and this inhibition effect on NHK was not related to the adhesion ability of the cells.

The attachment of keratinocytes to extracellular matrix proteins such as fibronectin and vitronectin is generally mediated through integrins. The β1 integrin subunit, which forms part of the receptor for fibronectin, is believed to be particularly important in matrix-cytoskeletal interactions and the regulation of keratinocyte receptor distribution. Studies on *Porphyromonas gingivalis* have shown that culture supernatants from the strains W50 or W83 caused cell detachment, degraded cell-associated fibronectin network and produced the loss of α5 and β1 integrin subunits

on human gingival fibroblasts *in vitro* (Scragg *et al*, 1996; 1999). These effects were mediated by proteases in the culture supernatant. However, the receptor for vitronectin (αv and β3 integrin subunits) was not disrupted. In *S. aureus*, extracellular proteases are produced during the post-exponential phase of growth. Major proteases produced by *S. aureus* include staphylococcal serine protease (V8 protease), metalloprotease (aureolysin), and cysteine proteases. It was considered that the decreased keratinocyte migration might be associated with the loss of integrin expression mediated by staphylococcal proteases. However, flow cytometry revealed that the level of surface expression of α5, β1, αv, and β6 integrin subunits on keratinocytes was not altered by the culture supernatants from 8325-4 over 18 hours of exposure. This suggests that the inhibitory components are less likely to be proteases and that the decreased cell movement caused by staphylococcal secreted products does not involve the loss of cell surface integrin expression.

The present data further show that pretreatment of 8325-4 and LS-1 stationary phase culture supernatants by heating completely abrogated their inhibitory effect on cell migration, whereas pretreatment with α_2-macroglobulin protease inhibitor had no rescue effect. These findings indicate, and confirm the above finding, that the inhibitory effects caused by *S. aureus* culture supernatant are not mediated by proteases. By SDS-PAGE analysis, studies of 8325-4 grown in BHI have shown that most exo-proteins were produced during the transition between late post-exponential (6 hours) and stationary (8 hours) phases (Chan and Foster, 1998a; 1998b). In addition, the most prominent protein in the 8325-4 profile was α-toxin (Chan and Foster, 1998a). For LS-1 grown in BHI, two most prominent proteins secreted were TSST-1 and β-toxin. In the present study, the relative amount of α-toxin and β-toxin presented in the supernatants of strains 8325-4 and LS-1 grown in THB for 18 hours was determined by measuring hemolytic activity against rabbit and sheep erythrocytes respectively. Strain LS-1 produced similar amounts of α-toxin to 8325-4, although the hemolytic titer was quite low (titer 32). Hemolytic activity against sheep red blood cells (β-toxin) in LS-1 was 4-fold higher than 8325-4. It is possible that the heat-labile nature of the secreted component responsible for cell migration inhibiton from 8325-4 and LS-1 is α- or β-toxin. It will be interesting to determine the involvement of α-toxin for these negative effects on keratinocytes using culture

supernatant prepared from an isogenic mutant strain in which the α-toxin gene is inactivated.

In conclusion, the present study shows that there were strain- and growth phase-dependent effects of the culture supernatants from *S. aureus* with regard to the detachment, viability, adhesion and migration of human keratinocytes. These may be due to variation in the virulence properties of different strains and growth phases. The culture supernatants from *S. aureus* strain NCTC6571, in which α- and β-toxin could not be detected, did not have any deleterious effects on keratinocytes. The secreted products from the stationary phase *S. aureus* strains 8325-4 and LS-1 inhibited keratinocyte migration. However, surface expression of fibronectin-binding integrins on keratinocytes was not altered. The inhibitory factor was heat-sensitive and protease inhibitor-resistance. This inhibitory activity may have a role to play in tissue damage and delayed healing of infected wounds.

Chapter 7

Effect of Staphylococcal α-toxin on Human Keratinocytes

7.1 Introduction

Staphylococcus aureus can produce a wide variety of exoproteins that contribute to its ability to colonize and cause infection in humans. Most *S. aureus* strains secrete a group of enzymes and cytotoxins including coagulase, staphylokinase, lipase, hyaluronidase, protease, α-, β-, γ-, and δ-hemolysins, TSST-1, enterotoxins, leukocidin, exfoliative toxins, and β-lactamase (Projan and Novick, 1997). Alpha-hemolysin (α-toxin) is produced by most strains of *S. aureus*. It is considered a major cytotoxin and is extremely potent. It is a pore-forming hemolytic toxin that causes membrane damage to many types of mammalian cells. Monocytes (Bhakdi *et al*, 1989), lymphocytes (Jonas *et al*, 1994), endothelial cells (Suttorp *et al*, 1988), fibroblasts (Walev *et al*, 1994) and keratinocytes (Walev *et al*, 1993) are lethally damaged by low doses of α-toxin (<1 μg/ml). Alpha-toxin assembles on cell membranes to form transmembrane, heptameric pores of 1-2 nm in diameter permitting influx/efflux of low-molecular mass molecules of less than 4 kDa (Bhakdi and Tranum-Jensen, 1991). Alpha-toxin causes dermal necrosis when injected subcutaneously (Patel *et al*, 1987). The dermonecrotic action may cause the tissue damage associated with wound infections.

Another potent cytotoxin produced by *S. aureus* is β-hemolysin (β-toxin). Beta-toxin is produced in large amounts by a number of *S. aureus* strains, particularly animal strains (Bohach *et al*, 1997). It is actually an enzyme with the ability to degrade sphingomyelin, which is a phospholipid commonly found as a component of the outer leaflet of many eukaryotic cell membranes. The sphingomyelinase activity of β-toxin requires the presence of divalent ions, particularly Mg^{2+} (Marshall *et al*, 2000). Hydrolysis of sphingomyelin in the cell membrane results in increased permeability or renders the cells more susceptible to the action of other cytotoxins or enzymes. The role of β-toxin in disease is not clearly understood.

Studies described in Chapter 6 have shown that stationary phase culture supernatant from *S. aureus* strain 8325-4 inhibited proliferation, adhesion and migration of primary human oral keratinocytes (NHK) and immortalized epidermal keratinocytes. The inhibitory factors were sensitive to heat but were resistant to α_2-macroglobulin, a protease inhibitor. The aim of this study was to determine the role of α-toxin on the inhibitory activity of 8325-4 culture supernatant by using an α-toxin knockout strain (DU1090) of *S. aureus* 8325-4.

7.2 Materials and Methods

7.2.1 Keratinocytes

UP keratinocytes and NHK were used in the study. They were prepared and maintained as described in Section 2.2.

7.2.2 Preparation of *S. aureus* Culture Supernatants

Two strains of *S. aureus* were included in the study: 8325-4 and its isogenic mutant lack of α-toxin, DU1090 (reviewed in Section 2.9.1). Stationary phase culture supernatants from both strains were collected after 24 hours of cultures using the method described in Section 2.11. The numbers of bacteria obtained in the stationary phase for both strains were similar as determined by spectrophotometry using a standard curve relating bacterial count to optical density (see Appendix A). The production of α- and β-toxin by 8325-4 and DU1090 after 24 hours in culture was determined by measuring the hemolytic activity against rabbit (α-toxin) or sheep (β-toxin) erythrocytes as described in Section 2.12.

7.2.3 Protein Analysis for Detection of Alpha-toxin in Bacterial Culture Supernatant

Protein analysis using SDS-PAGE and matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS) for detection of α- or β-toxin was performed with freshly prepared culture supernatants from 18 hour cultures of both 8325-4 and DU1090.

7.2.3.1 Isolation of Secreted Proteins from Culture Supernatants

The secreted proteins in bacterial culture supernatant were precipitated with 100% trichloroacetic acid (Sigma®). Briefly, the bacterial culture supernatant was mixed 4:1 with 100% trichloroacetic acid and incubated with mixing at 4°C for 20 min. The extracted protein was collected by centrifugation at 14,000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended and washed once with 100 µl of 80% acetone (in water). Following further centrifugation at 14,000 rpm for 10 min at 4°C, the pellet was allowed to air dry before resuspending in reducing sample buffer (see Appendix A) and the proteins separated by SDS-PAGE.

7.2.3.2 SDS-PAGE and Protein Identification with Mass Spectrometer (MS)

The SDS-PAGE for determination of staphylococcal hemolysins in culture supernatants was carried out using stacking and running gels with 5 and 12% acrylamide (see Appendix A), respectively. The proteins were separated under constant current beginning at 30 mA per gel for 15 min, then dropping to 20 mA per gel for 40 min or until the electrophoresis was complete. Separated proteins on the gel were stained overnight with Brilliant Blue G-colloidal Concentrate (Sigma®) and destained in water.

To identify proteins of interest, the protein bands at approximately 35 kDa were sliced and subjected to in gel digestion with trypsin and the peptide fragments were extracted and analyzed by MALDI-TOF MS using the Voyager-DE™ PRO BioSpectrometry™ Workstation and the Data Explorer™ Software, version 3.2 (PerSeptive Biosystems Inc, Framingham, MA, USA).

7.2.4 Cell Viability Measurement

Cell viability was assessed by the ability of cells to produce a colored formazan product (metabolic activity). Subconfluent keratinocyte cultures were harvested and resuspended in fresh KGM. Suspensions of UP and NHK were plated in 96-well plates at a concentration of 10,000 cells per well. Keratinocytes were

allowed to settle and attach for 24 hours producing subconfluent cultures before the experiments. The cells were washed once with PBS and incubated in DMEM for 2 hours. Duplicate wells were then incubated with 0.01%, 0.1%, 1%, 5%, or 25% concentration of *S. aureus* culture supernatant diluted in DMEM for 3, 6, or 24 hours at 37°C. The number of viable cells in each well was determined by the MTS assay as described in Section 2.3 by adding 20 µl of the CellTiter 96® reagent in each well one hour before the indicated time without washing. The absorbance was recorded at 492 nm within one and half hours.

To observe changes in cell morphology, keratinocytes were plated in a 96-well plate and treated with *S. aureus* culture supernatants as described above. After 3, 6 or 24 hours of exposure, the cells in each well were fixed with 10% formalin and stained with 0.5% crystal violet. Cell morphology was observed wet with an inverted microscope (model Leica DM IRB) equipped with a Leica DC200 digital camera (Leica Microsystems Wetzlar GmbH, Germany).

7.2.5 Cell Adhesion Assay

Cell adhesion assays were performed in 96-well plates coated with fibronectin as described in Section 2.5. Keratinocytes in suspension were pre-incubated with 0.01%, 0.1%, 1%, 5%, or 25% concentration of *S. aureus* culture supernatant diluted in serum-free medium (SFM) for 30 min at room temperature before plating. The cells, in the presence of the *S. aureus* culture supernatant, were allowed to settle and attach for 2 or 24 hours at 37°C before gentle washing twice with PBS. The number of attached cells in each well was quantified by the MTS assay, and presented as percent of the control treatment in Todd Hewitt broth.

7.2.6 Measurement of the Influx of Propidium Iodide into *S. aureus* Culture Supernatant-treated Keratinocytes by Flow Cytometry

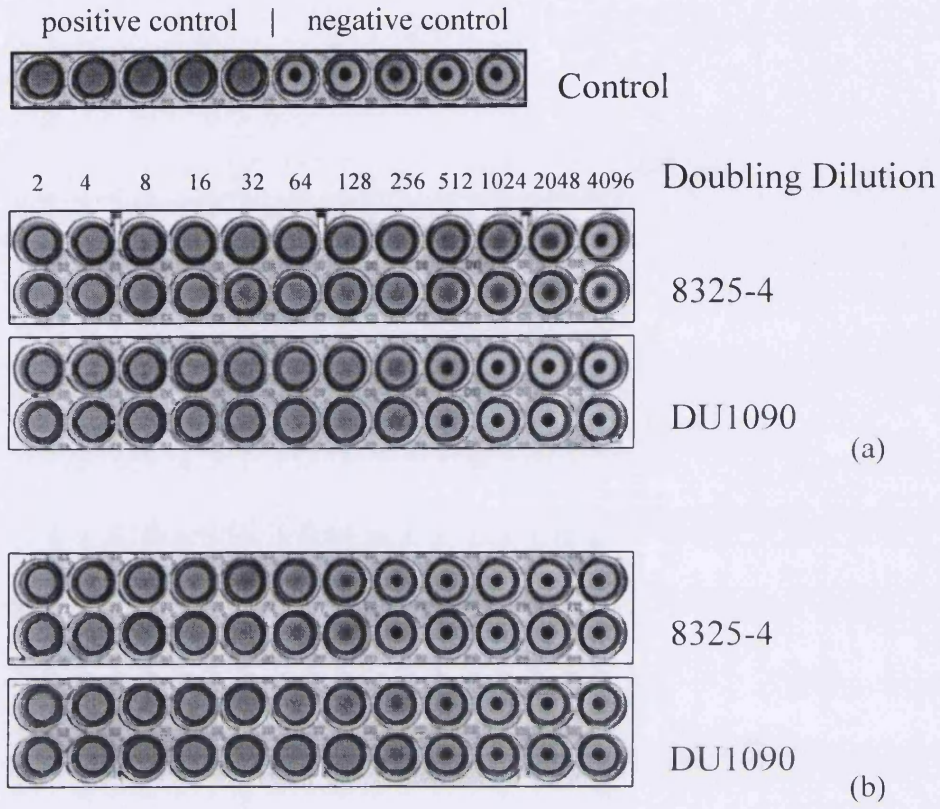
Because the intact membrane of live cells excludes a variety of charged dyes such as propidium iodide and trypan blue. Incubation with these dyes results in selective labeling of damaged or dead cells, while live cells show no, or minimal, dye uptake. After crossing the plasma membrane, propidium iodide binds to DNA and

dsRNA, and fluoresces (red) intensely. Subconfluent keratinocyte cultures were harvested and resuspended in fresh KGM. Suspensions of NHK and UP cells were aliquoted at approximately 500,000 cells per tube. The cells were washed once with SFM containing 0.5% BSA (SFM-0.5%BSA) before incubation with 0.01%, 0.1%, 1%, 5%, or 25% concentration of *S. aureus* culture supernatant diluted in SFM-0.5%BSA for 30 min at room temperature. After 30 min, cells were washed once with and resuspended in 10%FCS-PBS. Propidium iodide was added in each tube at a final concentration of 10 μg/ml, and the cells were left at room temperature for another 10 min before analysis by flow cytometry.

7.3 Results

7.3.1 Hemolytic Activities of Culture Supernatants from α-toxin Producing Strain 8325-4 and α-toxin Knockout Strain DU1090

Lysis of rabbit and sheep erythrocytes by *S. aureus* culture supernatants was used to measure the relative amount of α- and β-toxin present in the supernatants respectively. The results are given in Figure 7-1. The hemolytic activity with sheep erythrocytes from both wild-type α-toxin producing strain 8325-4, and isogenic α-toxin deficient strain DU1090, was similar. This indicates that the production of β-toxin is not affected by the defective gene for α-toxin in DU1090. Hemolytic titers for α-toxin from strains 8325-4 and DU1090 were 2,048 and 256 respectively. Comparison of the hemolytic titers with a similar study by Nilsson *et al* (1999) demonstrated a hemolytic titer from DU1090 of 64, the higher hemolytic activity (256) seen in the present preparation may be due to additive effects of other toxins and proteases in the supernatant. It is unlikely to be caused by recovery of α-toxin production by the mutant. This was confirmed by the absence of an approximately 34 kDa protein band from the SDS-PAGE which was present in 8325-4 culture supernatant (Figure 7-2). The band was identified to be staphylococcal α-toxin by MALDI-TOF MS. The 37 kDa protein band present in DU1090 culture supernatant was identified to be staphylococcal β-toxin.



S. aureus strain	Hemolytic titer	
	α-toxin	β-toxin
8325-4	2048	128
DU1090	256	256

Figure 7-1 *In vitro* hemolysin production by *Staphylococcus aureus* strains 8325-4 and DU1090. The stationary phase culture supernatant at 24 hours from each strain was titered for hemolytic activity against rabbit (a) or sheep (b) erythrocytes. The hemolytic titer is the highest dilution giving rise to lysis of rabbit (α-toxin) or sheep (β-toxin) erythrocytes. Control wells were incubated with water (positive control, five wells on the left) or PBS (negative control, five wells on the right).

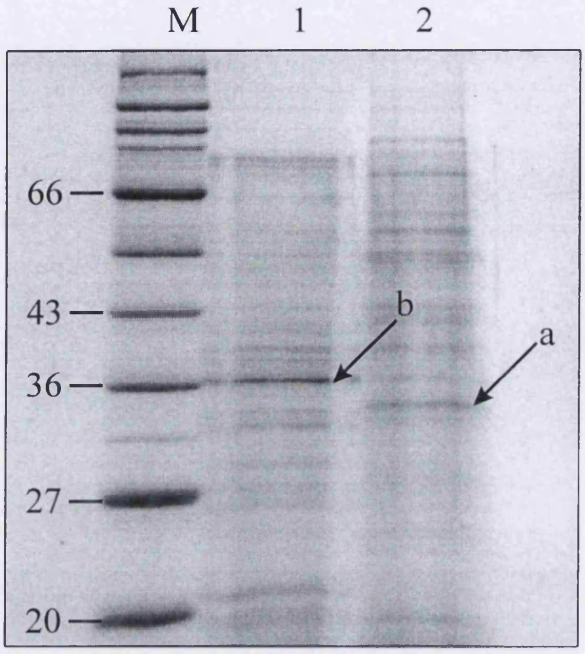


Figure 7-2 SDS-PAGE analysis of secreted proteins produced by staphylococcal α-toxin-producing strain 8325-4 (lane 2) and α-toxin-deficient mutant DU1090 (lane 1) at 18 hours stationary phase of growth in Todd Hewitt broth. Both lanes contained exo-proteins from equal volume of the culture supernatants. The molecular masses of protein standards (lane M, in kilodaltons) are shown. The protein bands a and b were cut and identified to be staphylococcal α-toxin and β-toxin respectively by MALDI-TOF mass spectrometry.

7.3.2 Comparison of Cell Morphology Changes and Cytotoxicity by Culture Supernatants from α-toxin Producing Strain 8325-4 with α-toxin Knockout Strain DU1090

Figure 7-3 (left column) shows that 5% and 25% 8325-4 culture supernatants were cytotoxic to UP keratinocytes, as demonstrated by cell shrinkage, rounding and detachment. These changes were observed as early as 3 hours. The slight changes of cell morphology observed at 3 hours with 1% 8325-4 culture supernatant were not observed at 24 hours (data not shown).

At concentrations ≤ 5%, culture supernatant from DU1090 was not cytotoxic to UP cells. Figure 7-3 (right column) shows that, at doses ≤ 5% concentration, no cell shrinkage or rounding was observed. However, membrane rupture and cell detachment were observed after exposure to 25% culture supernatant, as characterized by nonstained "phantom-like" cells. A similar effect was seen when relative cell viability was determined.

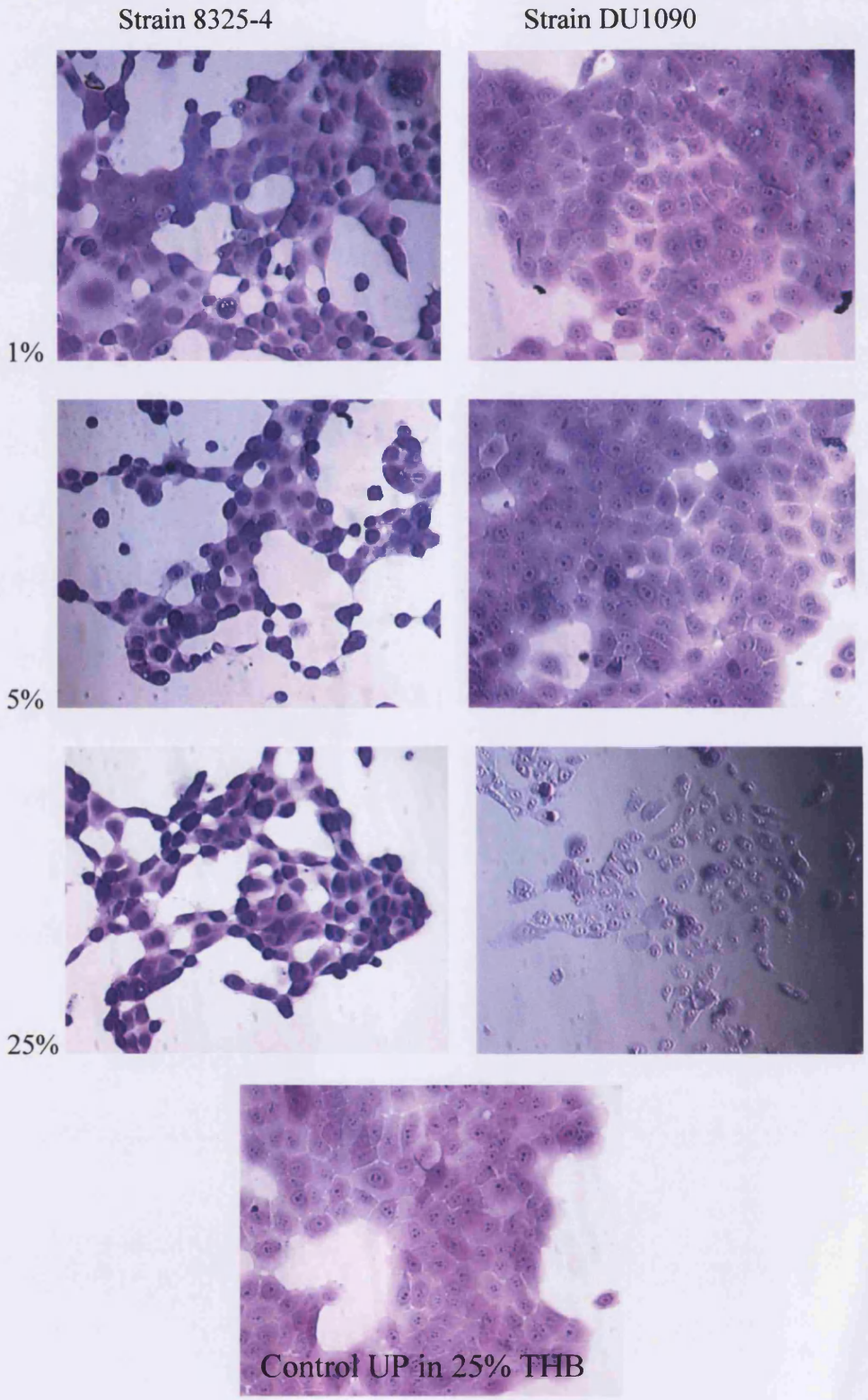


Figure 7-3 Photomicrographs of UP keratinocytes after 3 hours of exposure to various concentrations of the culture supernatant prepared from α–toxin producing *S.aureus* strain 8325-4 or α–toxin deficient mutant DU1090 (crystal violet stain, original magnification: x200).

To determine the viability of keratinocytes, the ability of cells to produce the colored MTS formazan product was measured. Figures 7-4 and 7-5 show that, at high concentration (25%), culture supernatants from both 8325-4 and DU1090 induced cell death to the same extent. At lower concentrations, culture supernatant from α-toxin producing strain 8325-4 was more cytotoxic than that which lacked the toxin. Thus 5% 8325-4 culture supernatant reduced UP cell viability by approximately 80% at 24 hours, whereas DU1090 culture supernatant reduced cell viability by approximately 30% of the controls (Table 7-1). NHK viability after 24 hours exposure to the 5% culture supernatant from strains 8325-4 and DU1090 was approximately 50% versus 77%.

Table 7-1 Effect of culture supernatant (CS) from α-toxin producing strain 8325-4 and isogenic α-toxin deficient strain DU1090 on the viability of immortalized (UP) and primary (NHK) keratinocytes examined by the MTS assay at 24 hours.

Dilution of CS	MTS assay (% viable cells)		
	strain 8325-4	strain DU1090	*p-value*
UP			
0.1%	102.97 ± 8.93	103.10 ± 10.33	NS
1%	80.63 ± 18.22	95.27 ± 9.84	NS
5%	19.33 ± 4.03	67.86 ± 6.38	*< 0.001*
25%	33.93 ± 9.12	34.70 ± 4.09	NS
NHK			
0.1%	95.24 ± 8.53	99.37 ± 1.19	NS
1%	74.93 ± 5.37	86.30 ± 4.23	NS
5%	49.95 ± 2.30	77.50 ± 0.39	*0.03*
25%	27.89 ± 1.64	42.66 ± 11.10	NS

Control cells were treated with the same dilutions of Todd Hewitt broth and represented the 100% values in each experiment. The values represent mean ± standard deviation of three independent experiments performed in duplicate. Statistical significance (p-value) of the means between groups was shown. NS, not significant.

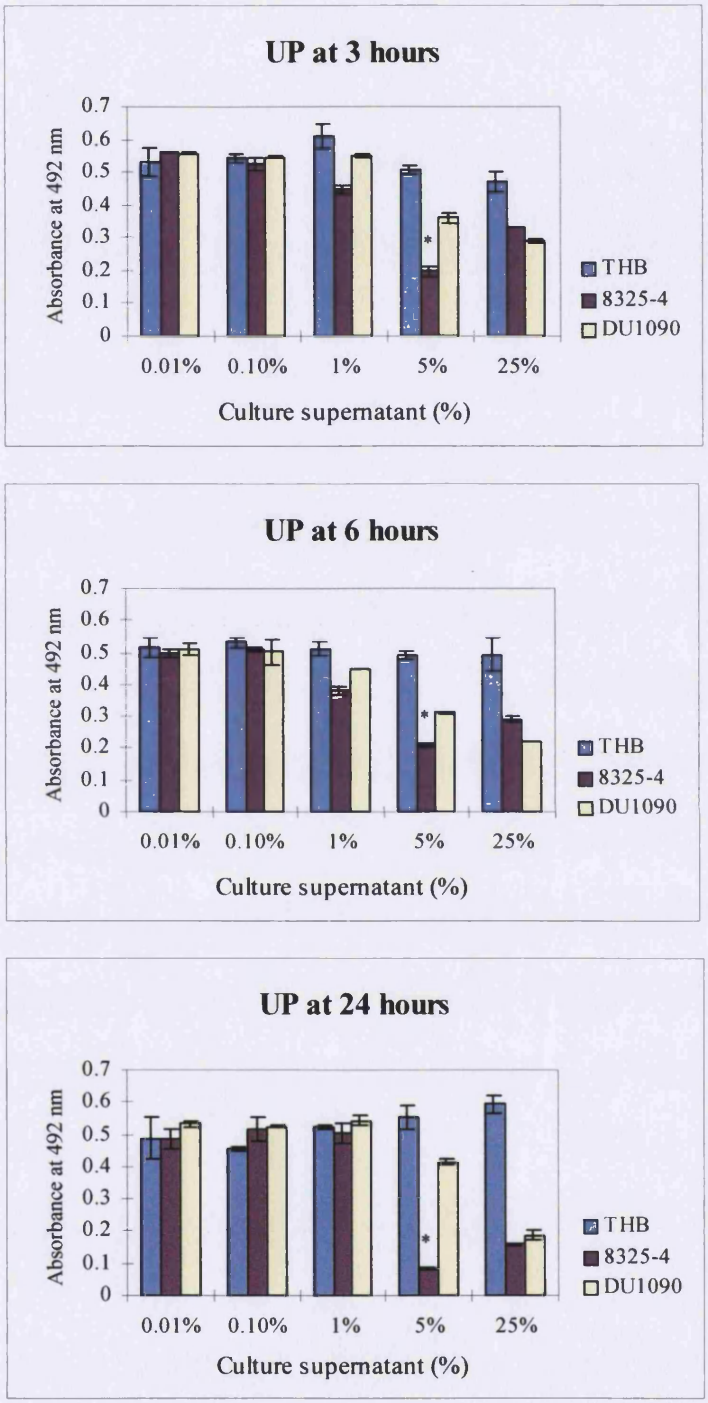


Figure 7-4 Comparison of the cytotoxic effect of culture supernatants from strains 8325-4 and DU1090 on UP keratinocytes. Relative cell viability was determined by the MTS assay. The plate was read on a microplate reader using a test wavelength of 492 nm. Data are expressed as mean ± standard deviation from a representative experiment, in duplicate. *, significantly different ($p < 0.05$) between 8325-4 and DU1090.

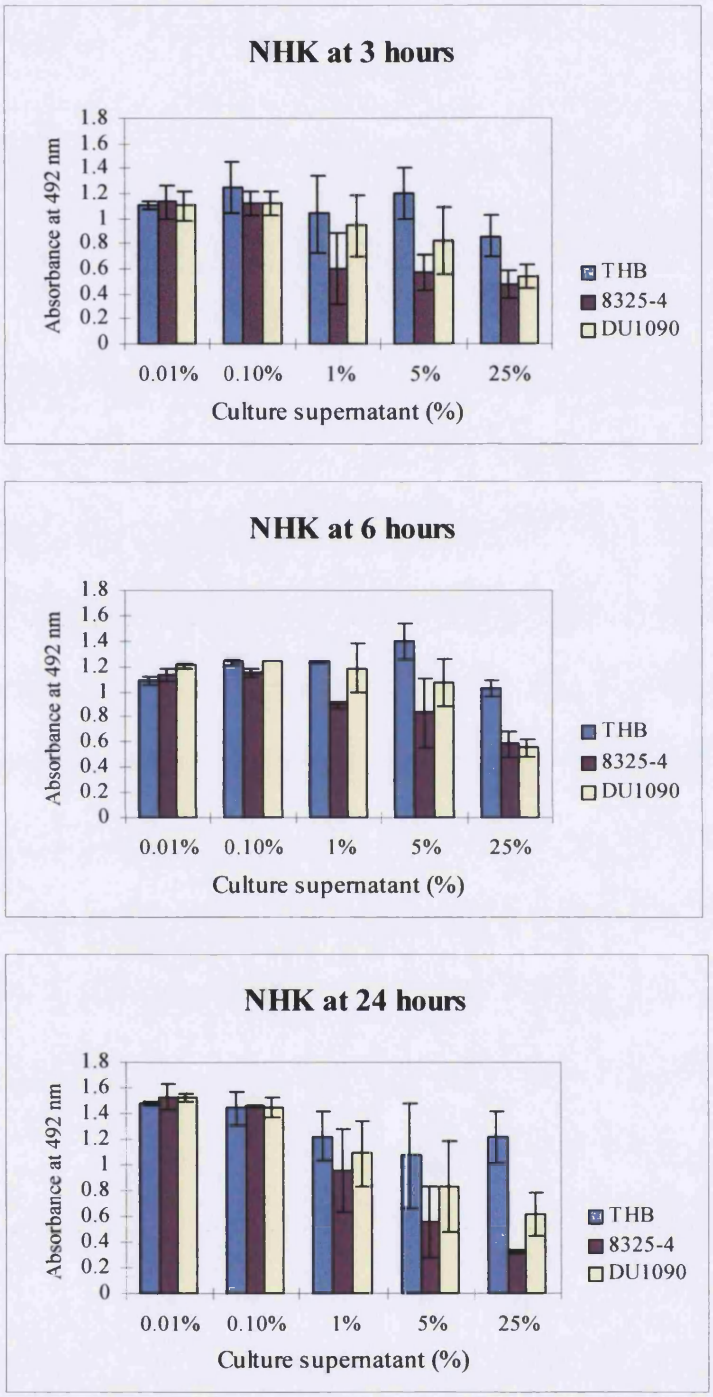


Figure 7-5 Comparison of the cytotoxic effect of culture supernatants from strains 8325-4 and DU1090 on NHK. Relative cell viability was determined by the MTS assay. The plate was read on a microplate reader using a test wavelength of 492 nm. Data are expressed as mean ± standard deviation from a representative experiment, in duplicate.

7.3.3 Comparative Effect of Culture Supernatants from α-toxin Producing Strain 8325-4 and α-toxin Knockout Strain DU1090 on Keratinocyte Adhesion

Cell attachment is necessary for spreading, migration and eventually replication of epithelial cells. Culture supernatants from 8325-4 and DU1090 inhibited cell adhesion in a dose-dependent pattern, and both abolished cell adhesion at 25% concentration. At lower concentrations, however, the inhibitory effect of α-toxin deficient strain DU1090 culture supernatant on cell adhesion was less than that of α-toxin producing strain 8325-4 at both 2 and 24 hours. Figure 7-6 shows that, at concentration as low as 1%, culture supernatant from strain 8325-4 reduced UP keratinocyte adherence by approximately 80% of the controls, whereas at the same dilution, DU1090 reduced the adhesion by only 20% to 30% at 2 hours and was similar to the control at 24 hours. Similar results were obtained with NHK.

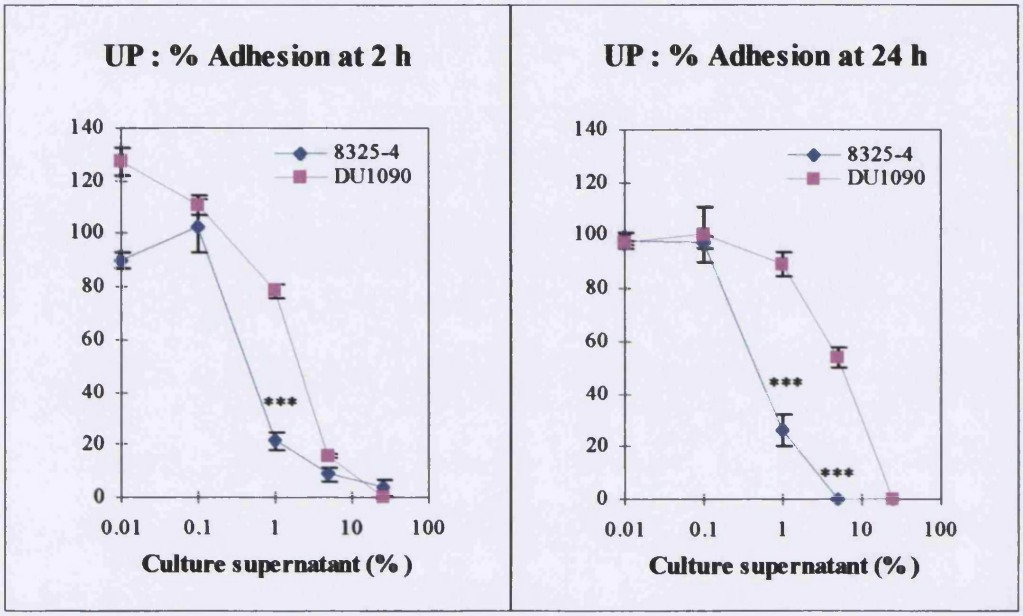
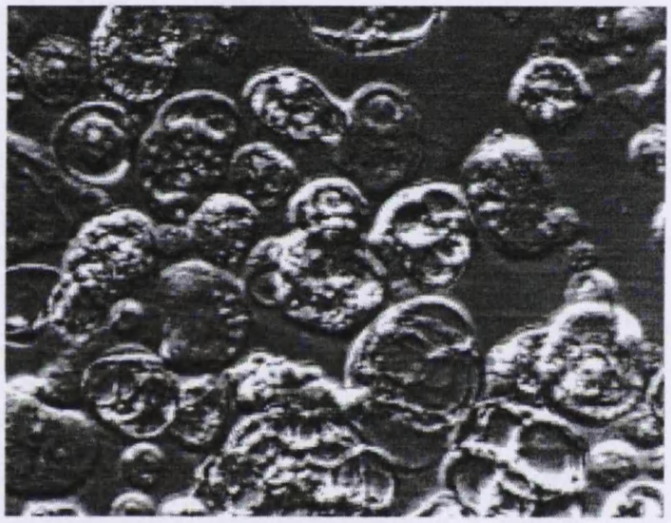
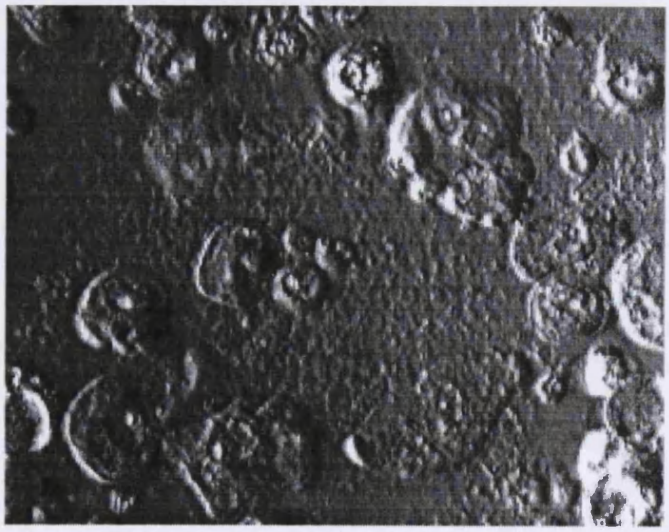


Figure 7-6 Comparative effect of culture supernatants from strains 8325-4 and DU1090 on the adhesion of UP (a) and NHK (b) at 2 and 24 hours in culture. Data are expressed as mean ± standard deviation from one experiment performed in triplicate. Control cells were treated with the same dilutions of Todd Hewitt broth and represented the 100% values in each experiment. Significant difference between groups at each dilution: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

While culture supernatants from both strains of *S. aureus* abolished keratinocyte adhesion at 25% concentration, the morphological changes in the non-adherent keratinocytes were different. The majority of keratinocytes incubated for 24 hours in 25% culture supernatant from α-toxin producing strain 8325-4 exhibited cell swelling with the absence of cell lysis (Figure 7-7a). However, there were signs of cell lysis and membrane rupture containing cellular and nuclear debris (Figure 7-7b) after incubation with culture supernatant from α-toxin deficient strain DU1090.



(a)



(b)

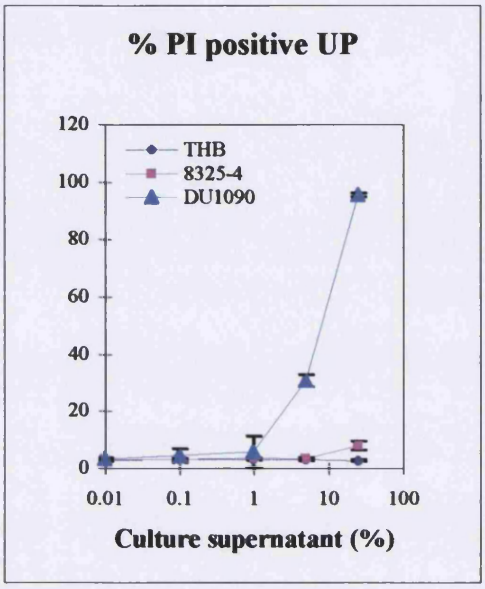
Figure 7-7 Phase contrast micrographs of primary keratinocytes (in suspension) after 24 hours incubation with 25% culture supernatant from α-toxin producing strain 8325-4 (a) or isogenic α-toxin deficient strain DU1090 (b). Original magnification: x100.

7.3.4 Culture Supernatant from α-toxin Knockout Strain DU1090 But Not α-toxin Producing Strain 8325-4 Causes Transmembrane Flux of Propidium Iodide

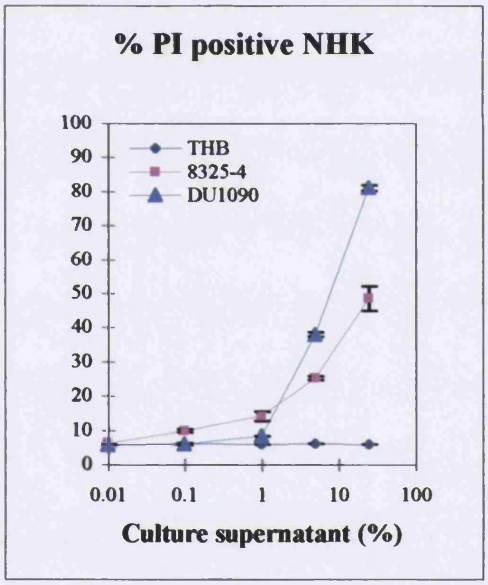
The studies in this Chapter have shown that morphological changes of the adherent and non-adherent keratinocytes induced by 8325-4 and DU1090 were different. Shrinkage and rounding of adherent keratinocytes, and cell swelling of non-adherent keratinocytes were characteristics of treatment with culture supernatant from α-toxin producing strain 8325-4, whereas membrane rupture and cell lysis occurred when cells were incubated with culture supernatant from α-toxin deficient mutant DU1090. In the next experiment, the nature of cell injury caused by culture supernatant from 8325-4 or DU1090 was further investigated by measuring the uptake of propidium iodide with flow cytometry. In pore-forming toxins, pore formation in nucleated cells can be detected by the influx of the dye markers. As shown in Figure 7-8a, only 3% of control intact UP cells (in THB) assumed red fluorescence of the propidium iodide after 30 minutes of incubation. UP treated with culture supernatant from DU1090 took up propidium iodide in a dose-response manner. At 25% concentration, more than 95% of UP were propidium iodide positive. In contrast, UP treated with 25% culture supernatant from 8325-4 did not take up significant amounts of propidium iodide. Using flow cytometry, one can quantify not only the amount of fluorescence emitted by individual flowing cells but also the relative cell size and granularity. When fluorescein-stained cells flow through the laser beam, three types of light scatter will be generated. Forward light scatter is generated by the light that passes through the cell and thus shadow of the cells appears. The size of the shadow is directly related to the cell size. Second, right-angle light scatter or side scatter is reflected by inner particles of the cell. The more granularity or complexity the cell has, the more light can be reflected and a high side scatter will be obtained. Third is the fluorescence light activated by the laser beam. The dot plots of forward scatter versus propidium iodide staining of treated and untreated UP cells are shown in Figure 7-9. There was a marked increase in forward light scatter parameter (relative cell volume) in UP cells treated with culture supernatant from strain 8325-4, but not strain DU1090, at a concentration as low as 0.1%, indicating the occurrence of cell swelling. These results indicate that the

membrane damage caused by culture supernatant from strain 8325-4 are small channels that allow cell swelling to occur but restrict diffusion of the propidium iodide.

Similar results were also obtained with primary keratinocytes NHK (Figures 7-8b and 7-10), however, approximately 25% and 45% of NHK took up propidium iodide when treated with culture supernatant from strain 8325-4 at 5% and 25% concentrations respectively. Cell swelling occurred after incubation with culture supernatant from strain 8325-4 as low as 0.1% dilution. Cell swelling of NHK was also observed after incubation with culture supernatant from strain DU1090, but it was minimal and occurred only at concentrations higher than 5%.



(a)



(b)

Figure 7-8 Comparative effect of culture supernatants from strains 8325-4 and DU1090 on transmembrane flux of propidium iodide (PI) on immortalized keratinocytes UP (a) and primary keratinocytes NHK (b). Data are expressed as the mean of percent of propidium iodide positive cells determined by flow cytometry ± standard deviation from 3 separate experiments. Cell suspensions incubated with Todd Hewitt broth (THB) served as controls.

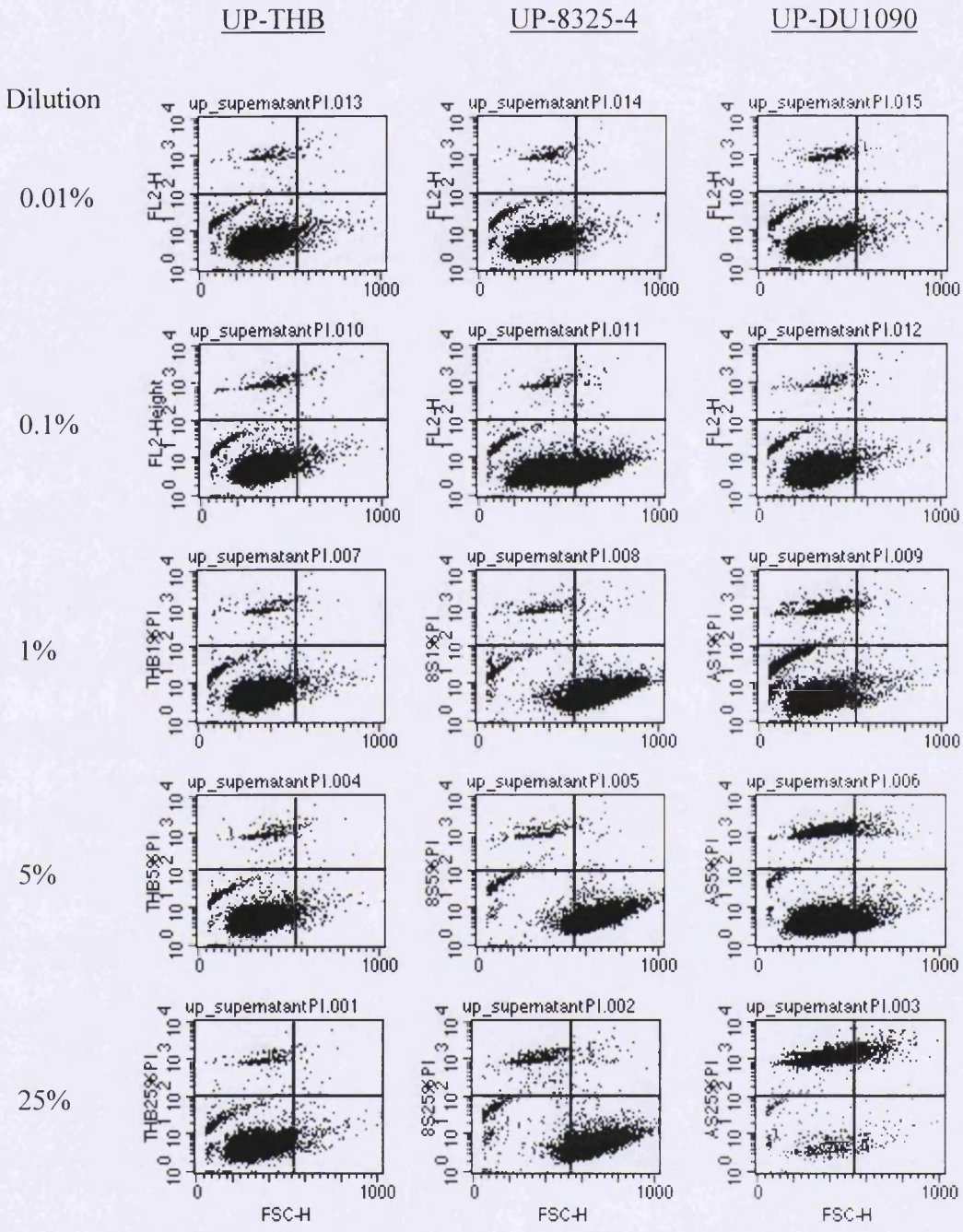


Figure 7-9 Dot plots displaying forward scatter versus propidium iodide staining of UP cells after exposure to various concentrations of culture supernatant from strain 8325-4 (middle column) or strain DU1090 (right column) for 30 minutes. Control cells were incubated with Todd Hewitt broth (THB, left column). Propidium iodide was added at a final concentration of 10 μg per ml for 10 minutes before analysis by flow cytometry.

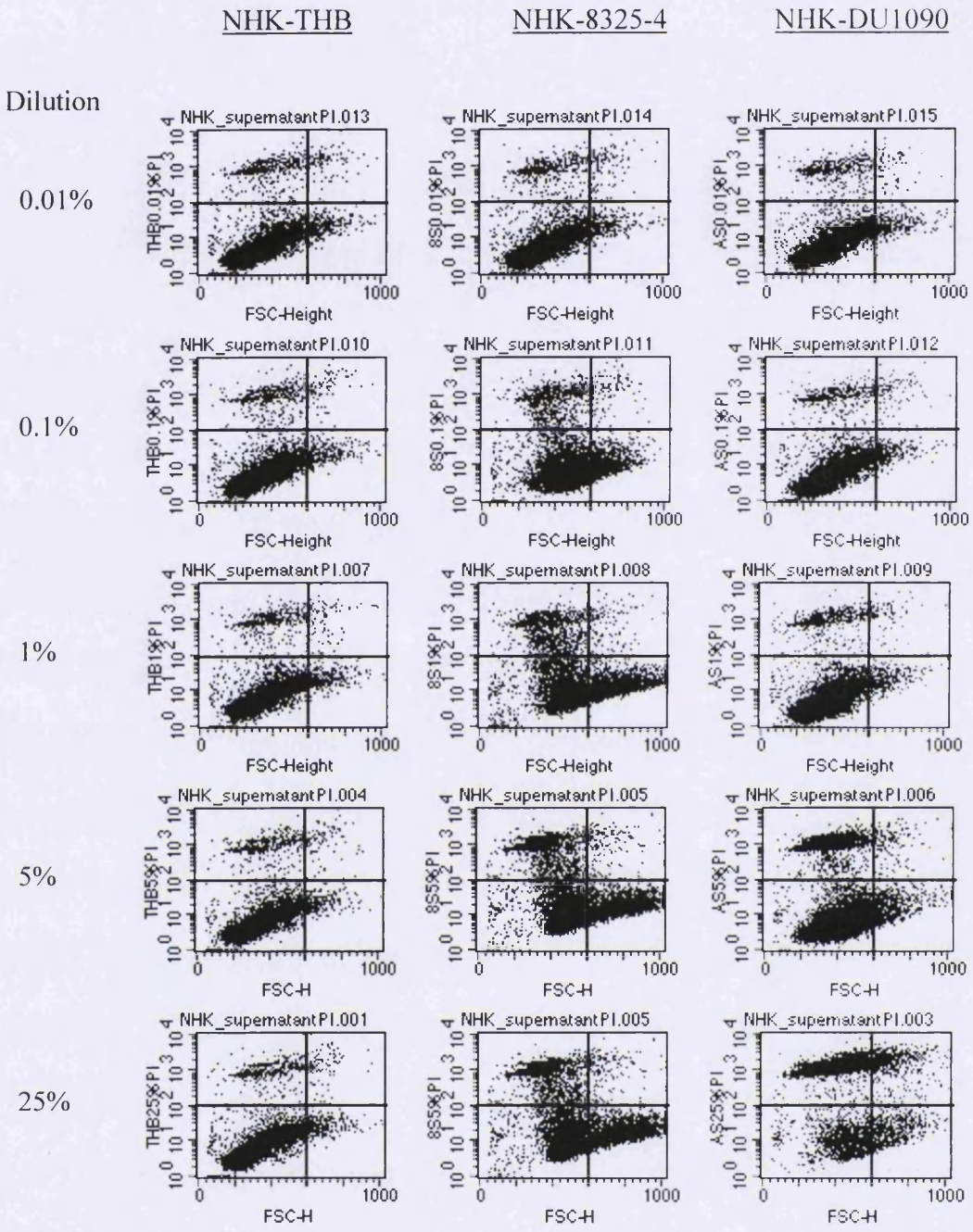


Figure 7-10 Dot plots displaying forward scatter versus propidium iodide staining of primary keratinocytes (NHK) after exposure to various concentrations of culture supernatant from strain 8325-4 (middle column) or strain DU1090 (right column) for 30 minutes. Control cells were incubated with Todd Hewitt broth (THB, left column). Propidium iodide was added at a final concentration of 10 μg per ml for 10 minutes before analysis by flow cytometry.

7.4 Discussion

Staphylococcus aureus eludes the host defenses and damages tissues by producing numerous virulence factors. Experiments in animal models indicate that α-toxin is an important virulence factor that is responsible for an increased severity of disease or death (Hume *et al*, 2001; Kielian *et al*, 2001; Dajcs *et al*, 2002). In a corneal keratitis model, it has been reported that the α-toxin producing strain 8325-4 caused significantly more extensive corneal erosions and severe inflammation than the α-toxin deficient mutant DU1090 (Hume *et al*, 2001). Alpha-toxin is a powerful pore-forming and cytolytic toxin, which is able to cause cell death in a variety of human cells including platelets, monocytes, endothelial cells and keratinocytes. The present study shows that culture supernatants from both α-toxin positive and negative strains of *S. aureus* were cytotoxic to keratinocytes causing cell death and detachment, and inhibited cell adhesion. However, the effect of culture supernatant from α-toxin producing strain 8325-4 was greater than that from DU1090 which lacked the toxin. At high concentrations (25%) there was no difference between strains on cell viability and adhesion. However, at lower concentrations (5% or less) the effect of culture supernatant from strain DU1090 was obviously less than that of α-toxin containing supernatant from 8325-4. This suggests that α-toxin is responsible for most of the damage to keratinocytes caused by *S. aureus* 8325-4. Study of culture supernatant prepared from *S. aureus* strain M60 and its α-toxin deficient mutant DU5789 with bovine mammary secretory epithelial cell monolayers also showed the decreased cytotoxic effect of the culture supernatant that lacked the α-toxin (Cifrian *et al*, 1996a). Recent studies have shown that, in the presence of α-toxin (as low as 6.0 nM), the EGF-receptor is unable to retain its phosphorylation signal in A431 carcinoma cells, and the process is irreversible (Vandana *et al*, 2003).

The present study has shown that inactivation of the α-toxin gene of *S. aureus* did not totally abolish the cytotoxic effect of *S. aureus* 8325-4 on keratinocytes. It is possible that the deleterious effects of α-toxin knockout strain DU1090 are due to other remaining toxins present in the culture supernatant, such as β-toxin. This is supported by the observation that cell morphology changes and the type of cell damage caused by culture supernatants from strains 8325-4 and DU1090

were different. Moreover, the relative amount of β-toxin in culture supernatant from strain DU1090 was slightly higher than from the parent strain 8325-4, with the hemolytic titers of 256 and 128 respectively. Beta-toxin has been identified as an enzyme (sphingomyelinase C) that specifically cleaves sphingomyelin, and as a remarkably potent monocytotoxic agent (Walev *et al*, 1996). The primary lesion induced by β-toxin in monocyte membranes is believed to be the generation of small permeability defects because influx of trypan blue occurs in the absence of lactate dehydrogenase efflux (Walev *et al*, 1996). Commercial β-toxin preparations from *S. aureus* have been shown to degrade sphingomyelin in the membrane of fibroblasts (Slotte and Bierman, 1988; Byers *et al*, 1992) without affecting viability (Walev *et al*, 1996). Fibroblasts incubated for 2 hours at 37°C with 1 U (enzymatic unit) of sphingomyelinase per ml (~20 hemolytic unit) remained trypan blue negative, and depletion of ATP was not observed (Walev *et al*, 1996). However, β-toxin has been reported to have a cytotoxic effect on bovine mammary epithelial cells, and exhibit an additive effect with α-toxin causing cell death, as determined by propidium iodide incorporation, when used at 32 hemolytic units per 100 μl (Cifrian *et al*, 1996a). The difference in the effect of β-toxin on fibroblasts and bovine mammary epithelial cells may be due to different doses used in those studies.

Although culture supernatants from both α-toxin positive and negative strains caused cell death and inhibited cell adhesion, it is believed that the component responsible for the effect from strain 8325-4 is mainly α-toxin. This belief is based on the finding that culture supernatant from α-toxin producing strain 8325-4 caused cell swelling without influx of propidium iodide. This effect is consistent with the observation that low doses (1 μg/ml) of purified α-toxin from *S. aureus* produces small plasma membrane pores on keratinocytes allowing only small molecules and ions such as monovalent cations to pass through (Walev *et al*, 1993). Cell lysis was not observed even after 24 hours of incubation of NHK with 25% culture supernatant from 8325-4. These results suggest that α-toxin is the major virulence factor secreted by *S. aureus* strain 8325-4 which inhibited keratinocyte proliferation, adhesion, and migration.

In the absence of α-toxin, culture supernatant from strain DU1090 caused immediate influx of propidium iodide into the cells, and induced cell lysis at 24 hours. This indicates that larger permeability defects occur in the absence of α-toxin, and allow large molecules and ions to pass through the cell membrane resulting in colloid-osmotic cell lysis. In addition, hydrolysis of sphingomyelin of the cell membrane by β-toxin may render the cells more susceptible to the lytic action of other toxins leading to cell death. The observation that the type of cell membrane damage changed from large permeabilized defects with cell lysis (in the absence of α-toxin) to very small pore formation without propidium iodide influx (in the presence of α-toxin) indicates that the activity of α-toxin in the culture supernatant of the parental strain is remarkably potent. However, the inactivation of a gene does not necessarily mean that all the other virulence factors are produced in the same manner as the isogenic parent. It would be interesting to see if adding a small amount of purified staphylococcal α-toxin to the culture supernatant from strain DU1090 would mimic the α-toxin-induced cellular defects observed in 8325-4.

In summary, this *in vitro* study shows that α-toxin was an important virulence factor that was responsible for increased cell death, and a decrease in the attachment and migration of keratinocytes. The activity of α-toxin was remarkably potent. Therefore, in the presence of α-toxin, the generation of very small permeability defects occurred leading to cell swelling without propidium iodide influx. In the absence of α-toxin, larger permeabilized cell membrane damage occurred leading to influx of propidium iodide and cell lysis.

Chapter 8
General Discussion and Conclusions

8.1 Introduction

Staphylococcus aureus is commonly found in the nasal cavity and on the skin (Kluytmans *et al*, 1997). Isolation of *S. aureus* from the mouth has been reported in approximately 24% of healthy adults and 64% of healthy children (Jackson *et al*, 1999). On tissue examination, *S. aureus* is found mainly in extracellular spaces. It can cause both localized infections (*e.g.* angular cheilitis and soft tissue abscesses) and life-threatening diseases (*e.g.* infective endocarditis and septic shock). *S. aureus* is also a common surface wound pathogen. Infections generally result from the ability of *S. aureus* to colonize host tissue surfaces and elaborate toxins and enzymes that destroy tissue and protect the bacteria from the host immune response.

8.2 Adherence of *S. aureus* to Human Keratinocytes

Adherence and colonization of host tissues is a common initial step in the pathogenic process of many infectious diseases. The present study shows that vitality of *S. aureus* was important for the adherence to human keratinocytes. *S. aureus* expresses a range of cell-wall associated proteins, known as MSCRAMMs, that promote adherence to host proteins such as fibronectin, fibrinogen, collagen, elastin, von Willebrand factor, vitronectin and bone sialoprotein. *In vitro* studies using *S. aureus* mutant strains deficient in the surface proteins have demonstrated that more than one surface adhesin of *S. aureus* plays a role in the adherence of *S. aureus* to human keratinocytes, because, although all the tested mutants showed reduced adherence, the reduction was only approximately 40% to 60% compared to the parental strains (Mempel *et al*, 1998 and the present study). These identified attachment factors to human keratinocytes were *S. aureus* FnBPs, clumping factor A or ClfA, and protein A. These data are in contrast with the studies from human endothelial cells (Peacock *et al*, 1999) and human airway epithelial cells (Mongodin *et al*, 2002) in which only mutant strains lacking FnBPs showed a reduction (over 80%) in adherence. It should be noted that adherence of *S. aureus* to primary oral keratinocytes was not affected by the lack of FnBPs (the present study). These data

taken together suggest that the interaction of *S. aureus* with human keratinocytes may be more complicated than with other cell types.

Environmental factors may also have an impact on bacterial colonization. For example, optimal adherence of *S. aureus* strain Newman to HaCaT keratinocytes was obtained at a pH between 7 and 8 and the highest adherent capacity was at 37°C (Mempel *et al*, 1998). In addition, adherence of *S. aureus* to buccal epithelial cells collected from smokers was higher than to the cells from non-smokers (El Ahmer *et al*, 1990).

The capacity of *S. aureus* to adhere to keratinocytes varied from strain to strain (Miyake *et al*, 1990 and the present study). Differentiation of cultured keratinocytes, induced by addition of 1.0 mM Ca^{2+} in the growth medium, has been shown to promote the adherence of some clinical isolates (3 out of 10) of *S. aureus* (Miyake *et al*, 1990). Adherence of *Streptococcus pyogenes* (impetigo strain 3722) was also demonstrated to be enhanced by keratinocyte differentiation (Darmstadt *et al*, 1998). These data suggest that host cell receptors for bacterial adherence may appear and/or increase with terminal differentiation. It was suggested that fibrinogen played a role in the binding of *S. aureus* to the horny layer of mouse skin as assessed by immunoelectron microscopy in an animal model (Kanzaki *et al*, 1996). Interestingly, recent *in vitro* studies have demonstrated that *S. aureus* fibrinogen-binding protein, clumping factor B (ClfB), promoted adherence of *S. aureus* to human cytokeratin 10 (K10) on both desquamated nasal epithelial cells and immortalized epidermal keratinocytes (O'Brien *et al*, 2002). K10 is a differentiation-associated marker expressed in the suprabasal compartment of stratified squamous epithelium (Chu and Weiss, 2002). The expression of K10 in any culture system, whether stratifying or monolayer, was reported to be on suprabasal differentiating keratinocytes only (Leigh *et al*, 1993). Immortalized or transformed keratinocytes and squamous carcinoma cell lines showed reduced stratification and contained a lower proportion of terminally differentiated cells than primary normal keratinocytes (Pei *et al*, 1991; Sugiyama *et al*, 1993; also see Chapter 3). Therefore, expression level of and/or proportion of cells expressing keratin K10 may be higher in primary cultured keratinocytes and that may have an effect on adherence and/or

internalization of *S. aureus* by the primary cells. It should be mentioned here that *S. aureus* used in the experiments described in this thesis were in the exponential phase of growth. Adherence of *S. aureus* to keratin has been demonstrated and is restricted to the bacteria from the exponential phase (O'Brien *et al*, 2002).

8.3 Human Keratinocytes as a Target for *S. aureus* Cytotoxins

Although several local and systemic diseases are caused by *S. aureus*, many more individuals are benignly colonized with no sign of disease (Projan and Novick, 1997). Colonization by *S. aureus* develops into clinically apparent infections usually after breaching of skin or mucosal barriers that allows the bacteria access to adjoining tissues or the bloodstream (Lowy, 1998). *S. aureus* produces numerous toxins that cause pore formation and induce proinflammatory changes in mammalian cells (*e.g.* cytotoxins and pyrogenic-toxin superantigens), and various enzymes that destroy tissue. Studies from atopic dermatitis lesions have shown that the density of colonized *S. aureus* correlated with cutaneous inflammation (Williams *et al*, 1990; Bunikowski *et al*, 2000). In addition, nine of 19 strains of *S. aureus* obtained from clinical lesions of atopic dermatitis and psoriasis produced α-toxin (Ezepchuk *et al*, 1996). Therefore, heavy colonization by *S. aureus* may lead to local tissue damage and infection.

Exposure of neutrophils to growth supernatant of *S. aureus* has been reported to induce apoptosis suggesting mediation by a bacteria-derived soluble factor (Lundqvist-Gustafsson *et al*, 2001). The secretory factor of *S. aureus* contributing to apoptosis of neutrophils was reported to be heat-sensitive. The present study shows that stationary phase culture supernatants from *S. aureus* strains 8325-4 and LS-1 inhibited keratinocyte migration. The inhibitory factor was heat-sensitive and protease inhibitor-resistance. In addition, staphylococcal cytotoxins *e.g.* α- and β-toxin were important virulence factors of *S. aureus* affecting human keratinocytes. Culture supernatants of *S. aureus*, in which activity of both α- and β-toxin were not detected, did not possess any deleterious effects on keratinocyte adhesion, migration or proliferation.

Alpha-toxin is produced by a high percentage of strains of *S. aureus*. Approximately 79% of *S. aureus* isolates from chronic, recurrent skin and respiratory tract infections produced α-toxin exclusively or in combination with other cytotoxins, while 8% of strains produced β-toxin (Slobodnikova *et al*, 1995). The activity of α-toxin is very potent and toxic to a wide range of mammalian cells. Levels of purified α-toxin as low as 1 μg/ml are lethal for keratinocytes (Walev *et al*, 1993), endothelial cells (Suttorp *et al*, 1988), monocytes (Bhakdi *et al*, 1989) and lymphocytes (Jonas *et al*, 1994; Tokura *et al*, 1997) in culture. Purified β-toxin of *S. aureus* has been demonstrated to have no cytotoxic effects on bovine mammary epithelial cells (^{3}H-thymidine assay) (Matthews *et al*, 1994) or human fibroblasts (trypan blue stain and intracellular ATP measurement) (Walev *et al*, 1996). Several studies in an animal model of *S. aureus* keratitis have shown that *S. aureus* strains, in which the α-toxin gene has been inactivated, were significantly less virulent than the parental strains (Hume *et al*, 2001; Dajcs *et al*, 2002). The present study shows that keratinocytes were killed by both secreted products from wild type 8325-4 and the α-toxin knockout strain DU1090 which contained no detectable α-toxin. The former caused cell swelling and the latter caused membrane rupture after exposure for 24 hours. The keratinocyte cell lysis observed may be caused by the activity of β-toxin alone or in synergy with other components in the culture supernatant of *S. aureus* strain DU1090. This observation is in accordance with studies showing that strains with single gene deletion (*e.g.* α-toxin or β-toxin) were not significantly less virulent than the parental strains (Kernodle and Kaiser, 1997; Nilsson *et al*, 1999) and that antiserum to α-toxin in combination with antiserum to β-toxin was the most effective inhibitor of cytotoxicity in *S. aureus* culture supernatants (Cifrian *et al*, 1996b).

8.4 Internalization of *S. aureus* by Human Keratinocytes

Similar to adherence, the capacity of *S. aureus* to be internalized by keratinocytes also varied from strain to strain (Figure 4-7). Difference in the capacity of different strains of *S. aureus* to be internalized was not related to their capacity to adhere to keratinocytes. As shown in Chapter 4, *S. aureus* strain LS-1 adhered to UP keratinocytes to a lesser degree than *S. aureus* strain 8325-4, but the level of internalization of LS-1 was approximately 10-fold higher than that of 8325-4. This

finding is consistent with studies from human osteoblast like cell line, MG-63 (Ahmed *et al*, 2001). For individual strains of *S. aureus*, however, lower internalization capacity as a direct effect of a decreased adherence capacity caused by any means or inhibitors, such as a recombinant protein of the FN-binding domain of *S. aureus* FnBPs, cannot be ruled out.

The work presented in this thesis has shown that the mechanisms of internalization of *S. aureus* by immortalized human keratinocytes (UP and squamous carcinoma cell lines) and primary oral keratinocytes (NHK) were different. The internalization of *S. aureus* by immortalized keratinocytes involved *S. aureus* FnBPs and host cell integrin α5β1, which is similar to previous studies from other cell types including human umbilical vein endothelial cells (Massey *et al*, 2001) and immortalized cell lines such as MG-63 cells (Ahmed *et al*, 2001; Nair SP, personal communication), 293 cells (Sinha *et al*, 1999) and HEp-2 cells (Dziewanowska *et al*, 2000). A recent study in HaCaT keratinocytes also showed reduced internalization of *S. aureus* strain lacking FnBPs compared to the parental strain (Mempel *et al*, 2002), however the role of α5β1 integrin was not investigated.

Internalization of *S. aureus* by primary oral keratinocytes was *S. aureus* FnBP-independent. Differences in the process of internalization of *S. aureus* by primary oral keratinocytes and immortalized keratinocytes are unlikely to be due to differences in the tissue from which the cells are derived (*e.g.* oral cavity versus skin), because carcinoma cell lines derived from oral tissues (H376 and H357 cells) and those derived from the epidermis (UP cells) internalized *S. aureus* by similar mechanism.

As discussed earlier in Section 8.2, it is possible that an interaction between *S. aureus* ClfB and host cell K10 occurs on primary keratinocytes and promotes internalization. Supportive data are: (1) ClfB of *S. aureus* binds to K10 on differentiating keratinocytes (O'Brien *et al*, 2002). (2) Keratin appears to be exposed on the surface of epithelial cells (Hembrough *et al*, 1995; Sajjan *et al*, 2000; 2002; Sojar *et al*, 2002). (3) Cell surface keratin can be internalized into an endocytic compartment (Ditzel *et al*, 1997). (4) Keratin participates in signaling processes

(Paramio *el al*, 1999). Further studies are required to identify and characterize bacterial and host factors responsible for the FnBP-independent adherence and internalization of *S. aureus* by primary (oral) keratinocytes.

8.5 Biological Activities of the FN-binding Domain of *S. aureus* FnBP

The work presented in Sections 5.3.5. to 5.3.7 has shown that a recombinant protein encompassing the D1-D4 repeat units of *S. aureus* FnBPB (rFnBPBD1-D4) inhibited endogenous FN polymerization by UP keratinocytes and also inhibited UP cell migration. Adhesion of the cells to immobilized FN and cell proliferation were unaffected. The D repeat units of *S. aureus* FnBPs bind exclusively to the N-terminus of FN, which participates in binding to cell surface FN assembly sites and in the self-assembly interactions. Accordingly, the *S. aureus* FN-binding proteins (and the rFnBPBD1-D4 protein) may have an influence on eukaryotic cells upon binding to FN or its fragments.

Fibronectin plays a critical role in different phases of wound healing (Ongenae *et al*, 2000) and has been demonstrated to be degraded in chronic wounds by proteases (Grinnell *et al*, 1992b; Marchina and Barlati, 1996). Proteolytic fragments of FN can display different biological activity from intact FN. The central cell-binding domain of FN can stimulate protease secretion by certain fibroblasts, even though the intact FN molecule does not (Werb *et al*, 1989). The 29 kDa amino-terminal FN fragment induces nitric oxide production from human articular chondrocytes, but the native FN does not (Gemba *et al*, 2002). Similarly, a fragment containing the N-terminus, but not the intact FN, displays potent chemotactic activity for human monocytes (Lohr *et al*, 1990). Interaction of the FN-binding domain of *S. aureus* FnBPs with these fragments of FN may have effects on cell behaviour.

8.6 Future Work

The present studies have demonstrated that, in contrast to immortalized keratinocytes, the adherence to and internalization of *S. aureus* by primary oral keratinocytes did not involve *S. aureus* FnBPs or the $\alpha5\beta1$ integrin. Future work is needed to identify and characterize the bacterial and host cell proteins responsible for the FnBP-independent binding and internalization of *S. aureus* by the primary oral

keratinocytes. The possible role of *S. aureus* ClfB and keratinocyte keratin 10 should be tested.

It was demonstrated that the rFnBPBD1-D4 protein inhibited migration of UP keratinocytes, but not primary oral keratinocytes, as determined by the haptotactic assay. Whether the migration inhibition effect is selective for immortalized and transformed keratinocyte cell lines needs to be clarified before making any conclusions. Experiments with other keratinocyte and squamous carcinoma cell lines are required as well as using different cell migration assays for the primary keratinocytes.

It was also demonstrated that the rFnBPBD1-D4 protein inhibited endogenous FN polymerization by UP keratinocytes. It is important to test whether the FN assembly inhibitory activity of the protein occurs with other cell types, fibroblasts in particular. Finally, interaction of the rFnBPBD1-D4 with FN fragments should also be investigated.

8.7 Conclusions

In conclusion, *S. aureus* can adhere to and be internalized by keratinocytes. With the exception of primary oral keratinocytes, the internalization process involved *S. aureus* FnBPs and keratinocyte integrin $\alpha5\beta1$. It should be noted that the difference in the response of primary cells and cell lines to *S. aureus* may have important implications for the interpretation and translation of *in vitro* studies to predict *in vivo* responses. Cytotoxins from *S. aureus*, such as α- and β-toxin, were important virulence factors of *S. aureus* affecting human keratinocytes. *S. aureus* may gain access to the deeper adjacent dermal tissues by fatal damage of keratinocytes with its cytotoxins. The FN-binding domain of *S. aureus* FnBPs (D1-D4 domain) affected UP cell migration and inhibited endogenous FN polymerization. Interaction of the FN-binding domain of *S. aureus* FnBP with intact and/or fragments of FN may influence cell behaviour.

Appendix A

Cultures, Antibiotics and Protein Purification

A-1 Culture of Keratinocytes

A-1.1 Culture Media

Transport Medium for Gingival Tissue

Dulbecco's Modified Eagle's medium (DMEM)	12.0	ml
10^4 IU/ml Penicillin/10^4 μg/ml Streptomycin	1.0	ml
250 μg/ml Fungizone (amphotericin B)	1.0	ml

Aliquot to 6 ml per tube, keep at 4°C, and use within 2 weeks.

Keratinocyte Growth Medium (KGM) (500 ml)

Dulbecco's Modified Eagle's medium	337.5	ml
Nutrient Mixture Ham's F-12	112.5	ml
Fetal calf serum (FCS)	50.0	ml
10 μg/ml Epidermal growth factor	0.5	ml
100 μg/ml Hydrocortisone	2.0	ml
10 mg/ml Insulin	0.25	ml
$1.8x10^{-2}$ M Adenine	5.0	ml
10^{-7} M Cholera toxin	0.5	ml
10^4 IU/ml Penicillin/10^4 μg/ml Streptomycin	5.0	ml
250 μg/ml Fungizone (amphotericin B)	5.0	ml

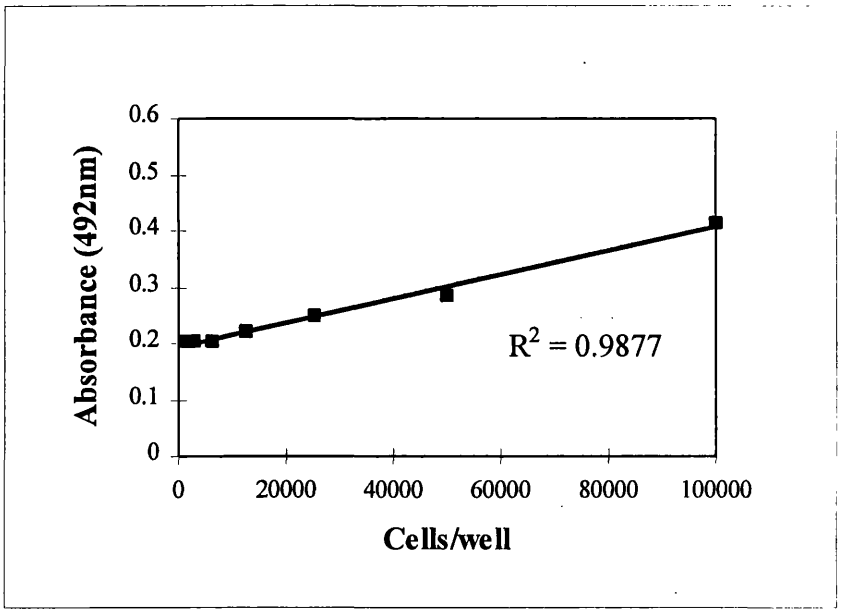
Filter the preparation with 0.22μm-pore size filter and keep at 4°C.

A-1.2 Determination of Cell Number

The number of cells was determined by using CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS assay) in a 96-well microplate. The MTS tetrazolium compound in the assay acts as an artificial hydrogen-acceptor substrate for dehydrogenase activity in the metabolically active cells. The bioreduced MTS

tetrazolium compound forms a colored formazan product which is soluble in tissue culture medium. This cytobiochemical assay can provide an indirect reflection of cell number, in that dehydrogenase activity usually relates to cell number. Assays are performed by adding a small amount of the CellTiter 96® reagent directly to culture wells, incubating for 1 hour and then recording absorbance at 492 nm with a 96 well plate reader. An example of effect of cell number on absorbance at 492 nm measured using the CellTiter 96® AQueous One Solution Assay is given below.

Cell number	1562.5	3125	6250	12500	25000	50000	100000
A492nm	0.205	0.2033	0.205	0.2223	0.2503	0.2837	0.4153



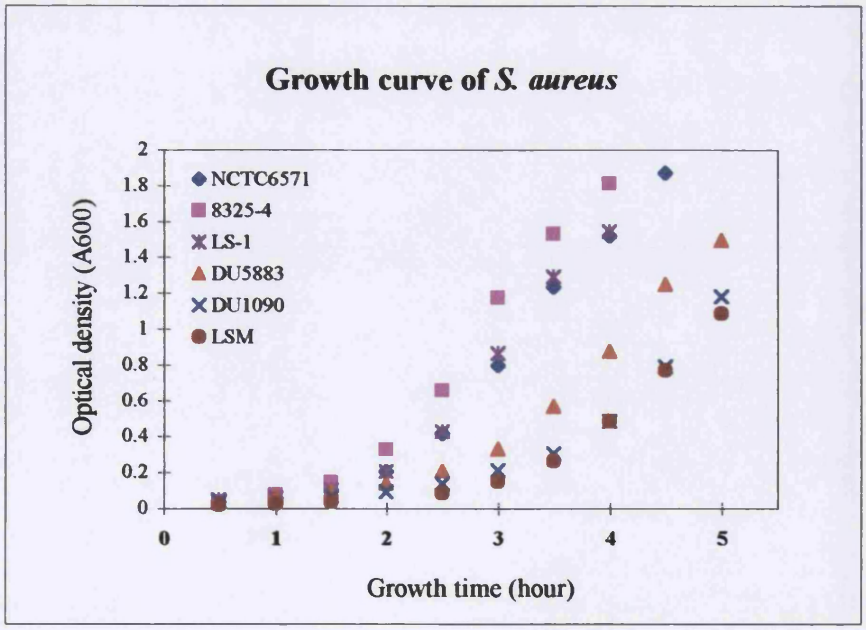
Various numbers of UP cells suspended in DMEM were added to the wells of a 96-well plate. The medium was allowed to equilibrate for 1 hour, then 20 μg/well of CellTiter 96® reagent was added. After 1 hour at 37°C in a humidified, 5% CO_2 atmosphere, the absorbance at 492 nm was recorded using an ELISA plate reader. Each point represents the mean ± standard deviation of three replicates. The correlation coefficient of the line was 0.9877, indicating that there was a linear response between cell number and absorbance at 492 nm. The background absorbance shown at zero cell/well was not substracted from these data.

A-2 Bacterial Culture

All *S. aureus* strains used in this thesis were grown in Todd-Hewitt broth (THB). *E. coli* containing plasmid for protein preparation were grown in Luria Broth (Miller's LB broth) purchased from Sigma®.

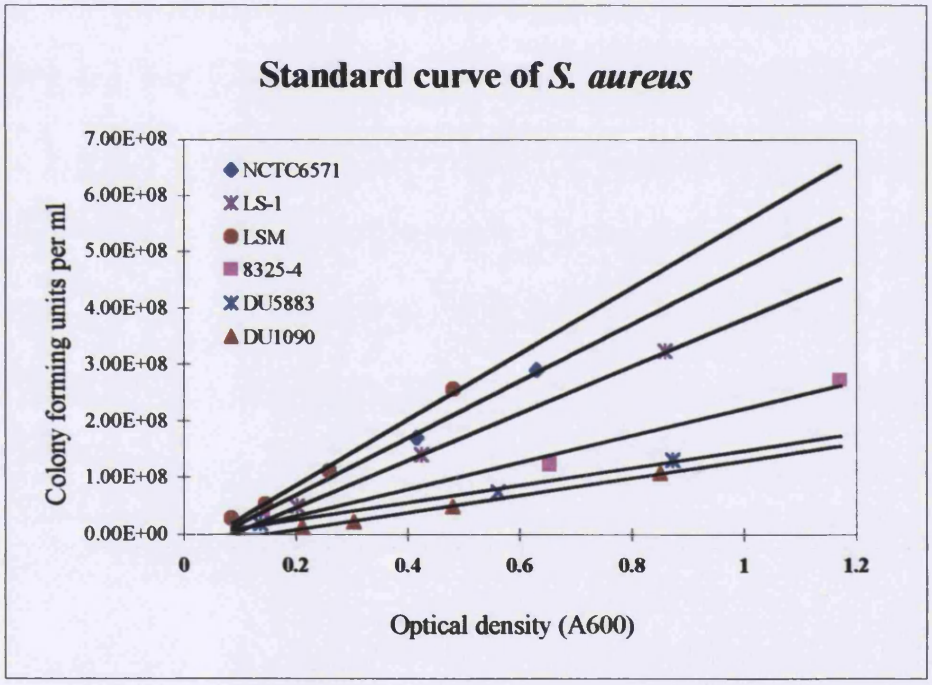
A-2.1 Bacterial Growth

Bacterial growth was determined by measuring turbidity of the bacterial growth medium at 30 minutes interval for 3 to 5 hours using a spectrophotometer. Overnight broth culture (1:250, v/v) was transferred to 150 ml fresh growth medium in a 250 ml-Erlenmeyer flask and incubated in a shaking incubator at 37°C. The turbidity of the growth medium was recorded using spectrophotometer set at wavelength 600 nm. The growth curve of *S. aureus* strains NCTC6571, 8325-4, DU5883, DU1090, LS-1 and LSM are shown below. All wild-type strains or mutant strains showed similar growth rate within the group. The mutant strains were grown in the presence of appropriate antibiotics at all time.



A-2.2 Determination of Bacterial Number (Standard Curve)

During the exponential growth, bacterial number was determined from at least three turbidity values. Viable bacterial counts were performed on 5% blood agar plates using a 10-fold dilution. The standard curve from *S. aureus* strains NCTC6571, 8325-4, DU5883, DU1090, LS-1 and LSM are shown below. The bacterial counts were plotted against the turbidity values. The correlation coefficient of each line was close to 1, indicating that there was a linear response between bacterial colony forming unit and the turbidity during the exponential phase of growth. Strain 8325-4 and its mutants (DU5883 and DU1090) had similar growth rate which was slower than that of strain LS-1 and its mutant LSM.



The correlation coefficient of strain

NCTC6571	$R^2 = 0.9977$
LS-1	$R^2 = 0.9999$
LSM	$R^2 = 0.9937$
8325-4	$R^2 = 0.9857$
DU5883	$R^2 = 0.9912$
DU1090	$R^2 = 0.9958$

A-3 Antibiotic Solutions

Concentration of stock solutions of antibiotics used in the study are shown below.

	Stock solution[a]	
	Concentration	Storage
Ampicillin	50 mg/ml in H_2O	-20°C
Erythromycin	5 mg/ml in ethanol	-20°C
Kanamycin	10 mg/ml in H_2O	-20°C
Spectinomycin	10 mg/ml in H_2O	-20°C
Streptomycin	10 mg/ml in H_2O	-20°C
Tetracyclin[b]	2 mg/ml in 50% ethanol	-20°C

[a]Stock solutions of antibiotics dissolved in H_2O are sterilized by filtration through a 0.22-micron filter. Antibiotics dissolved in ethanol need not be sterilized. Store solutions in light-tight containers.

[b]Magnesium ions are antagonists of tetracycline. Use media without magnesium salts for selection of bacteria resistant to tetracycline.

A-4 Protein Purification

Proteinase inhibitors, elution buffer, and reagents used in the preparation of rFnBPBD1-D4 protein were prepared as described below.

E-64 (L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane, MW = 357.4)

Make a stock solution of 10 mM E-64 by dissolving 1 mg of E-64 in 280 µl of PBS. Dispense the solution into 10 µl aliquots and store at -20°C.

IPTG (Isopropylthio-β-D-galactoside, MW = 238.3)

Make a stock solution of 1 molar IPTG by dissolving 1 g of IPTG in 4.2 ml of distilled H_2O. Dispense the solution into 0.85 ml aliquots and store at -20°C.

Leupeptin (Acetyl-Leu-Leu-Arg-al, FW = 475.6)

Make a stock solution of 10 mM Leupeptin by dissolving 1 mg of Leupeptin in 0.21 ml of distilled H_2O. Dispense the solution into 10 µl aliquots and store at -20°C.

Pepstatin A (FW = 685.9)

Make a stock solution of 10 mM Pepstatin A by dissolving 5 mg of Pepstatin A in 729 µl of DMSO or ethanol/acetic acid (90:10). Dispense the solution into 10 µl aliquots and store at -20°C. Make second stock solution of 1 mM Pepstatin A by adding 10 µl of 10 mM Pepstatin A into 90 µl of PBS. Dispense the solution into 10 µl aliquots and store at -20°C.

PMSF (Phenylmethyl-sulfonyl fluoride, MW = 174.3)

Dissolve PMSF in isopropyl alcohol or ethanol at a concentration of 0.0209 mg/ml (120 mM) and store at -20°C.

Caution: PMSF is extremely destructive to the mucous membranes of the respiratory tract, the eyes, and skin. It may be fatal if inhaled, swallowed, or absorbed through the skin. In case of contact, immediately flush eyes or skin with copious amounts of water. Discard contaminated clothing. PMSF is inactivated in aqueous solutions. The rate of inactivation increases with pH and is faster at 25°C than at 4°C. The half-life of a 20 µM aqueous solution of PMSF is about 35 minutes at pH 8.0. This means that aqueous solutions of PMSF can be safely discarded after they have been rendered alkaline (pH > 8.6) and stored for several hours at room temperature.

Imidazole (MW = 68.08)

1 M of imidazole can be prepared by dissolving 1.7 g of imidazole into 20 ml of de-ionized water (dH_2O). Adding 0.98 ml of 35% hydrochloric acid and adjust the volume to 25 ml with dH_2O. Prepare fresh each time before use. Imidazole at 250 mM in PBS was used as elution buffer in the preparation of rFnBPBD1-D4.

A-5 Protein Analysis

Tris Buffer

Weight Tris (hydroxymethyl aminomethane) according to the molarity needed and dissolve in dH_2O. Adjust the pH with concentrate hydrochloric acid (37%) and bring the volume to the desired amount.

Tris-glycine Eletrophoresis Buffer

25 mM Tris
250 mM glycine (eletrophoresis grade) (pH 8.3)
0.1% SDS

A 5x stock can be made by dissolving 15.1 g of Tris, 94 g of glycine, and 5 g of SDS in 900 ml of dH_2O. The pH will be about 8.3, and bring the volume to 1000 ml with dH_2O.

Resolving Gel for Tris-glycine SDS-polyacrylamide Gel Electrophoresis (15ml)

	12%	6%	
dH_2O	4.9	7.9	ml
30% acrylamide mix	6.0	3.0	ml
1.5 M Tris (pH 8.8)	3.8	3.8	ml
10% SDS	0.15	0.15	ml
10% ammonium persulfate	0.15	0.15	ml
TEMED	0.006	0.012	ml

Mix the components in the order shown. Add the last two reagents just before loading. Polymerization will begin as soon as the TEMED has been added. Without delay, swirl the mixture rapidly (avoid creating the air bubbles) and load the mixture solution into the gap between the glass plates.

5% Stacking Gel for Tris-glycine SDS-polyacrylamide Gel Eletrophoresis (5ml)

dH_2O	3.4	ml
30% acrylamide mix	0.83	ml
1 M Tris (pH 6.8)	0.63	ml
10% SDS	0.05	ml
10% ammonium persulfate	0.05	ml
TEMED	0.005	ml

Mix the components in the order shown. Add the last two reagents just before loading. Polymerization will begin as soon as the TEMED has been added.

Sample Loading Buffer (Laemmli, reducing)

	2x	5x
Tris (pH 6.8)	0.1 M	0.25 M
Glycerol	20%	50%
SDS	4%	10%
Mercaptoethanol	10%	25%
Bromophenol blue	trace amount to get blue colour	

Gel Fixation Solution

10% methanol (analytical grade) and 15% glacial acetic acid in dH_2O.

Coomassie Blue G-Colloidal Concentrate

Purchased from Sigma®.

Coomassie Brilliant Blue solution (100 ml)

Coomassie Brilliant Blue R250	0.25	g
Methanol: dH_2O (1:1 v/v)	90.0	ml
Glacial acetic acid	10.0	ml

Destain Solution

Destain solution after staining with Coomassie Blue G-Colloidal Concentrate is dH_2O. Destain solution after staining with Coomassie Brilliant Blue solution is a mixture of 45% methanol and 10% glacial acetic acid in dH_2O.

Appendix B

Biochemicals and Reagents

Products	*Manufacturer*	*Cat. No.*
ProtoGel®(30% Acrylamide (29% w/v acrylamide and 1% w/v N,N'-methylenebisacrylamide)	National Diagnostics	EC-890
Adenine	Sigma®	A-3159
Albumin, bovine (fraction V)	Sigma®	A-9418
Ammonium Persulfate	Sigma®	A-3678
B-PER® bacterial protein extraction reagent	Pierce	78248
Blood Agar Base No.2	Oxoid Ltd	CM271
5-BrdU	Sigma®	B-9285
Cholera Toxin	Sigma®	C-3012
Coomassie Blue G-Colloidal Concentrate	Sigma®	B-2025
DAPI	Sigma®	D-9564
Defined Keratinocyte-SFM	Gibco™	10785
Dimethyl Sulfoxide (DMSO)	Sigma®	D-4540
Dulbecco's Modified Eagle Media, low glucose	Gibco™	31885-023
E-64	Sigma®	E-3132
Epidermal Growth Factor (EGF)	Sigma®	E-4127
Fetal Calf Serum	PAA Laboratories	A15-649
Fibronectin, from human plasma	Sigma®	F-0895
Fibronectin Fragment, 30 kDa	Sigma®	F-9911
Hydrocortisone	Sigma®	H-0135
Insulin	Sigma®	I-1882
Imidazole	Sigma®	I-2399
IPTG (isopropylthio-β-D-galactoside)	BDH	437142L
Leupeptin	Sigma®	L-2884
Luria Broth (Miller's LB broth)	Sigma®	L-3522
α_2-Macroglobulin	Sigma®	M-6159
Ni-NTA Agarose	Qiagen Ltd	30210
Nutrient Mixture Ham's F-12, with L-glutamine	Gibco™	21765-029
Penicillin-Streptomycin	Gibco™	15140-122

Pepstatin A	Sigma®	P-5318
PMSF (phenylmethylsulfonyl fluoride)	Sigma®	P-7626
Polymyxin B Sulphate	Sigma®	P-1004
Protein Marker, broad range	New England Biolabs	P-7702
SDS (sodium dodecyl sulphate)	BDH	444464T
TEMED (N,N, N′,N′-tetramethylethylenediamine)	Sigma®	T-9281
Todd Hewitt Broth	Oxoid Ltd	CM189
Trichloroacetic Acid (TCA)	Sigma®	T-4885
Tris (hydroxymethyl) aminomethane	BDH AnalR	103156X
Triton X-100 (t-Octylphenoxypolyethoxyethanol)	Sigma®	X-100

Appendix C

Data for Figures in Chapters 3-7

Chapter 3

Data for Figure 3-9

Keratinocyte	Mean fluorescence intensity of integrin subunit			
	α5	β1	αv	β6
UP	2.93 ± 0.64	86.93 ± 12.2	19.07 ± 2.16	3.04 ± 1.18
NHK	29.81 ± 5.22	222.27 ± 20.2	97.43 ± 23.99	10.93 ± 0.7

Chapter 4

Data for Figure 4-5 (% of inoculum)

Adhesion to	*S. aureus* strain		
	NCTC6571	8325-4	LS-1
UP	10.91 ± 1.46[a,b]	43.70 ± 9.25	23.81 ± 2.36[c]
NHK	16.53 ± 2.94[d,e]	10.60 ± 1.95	7.20 ± 1.23[f]

[a], compared to the 8325-4, $p = 0.004$; [b], compared to the LS-1, $p = 0.001$;
[c], compared to the 8325-4, $p = 0.02$;
[d], compared to the 8325-4, $p = 0.045$; [e], compared to the LS-1, $p = 0.007$.
[f], compared to the 8325-4, $p = 0.06$.

Data for Figure 4-7 (% of inoculum)

Internalization by	*S. aureus* strain		
	NCTC6571	8325-4	LS-1
UP	0.003 ± 0.001[a,b]	0.163 ± 0.051	0.7 ± 0.11[c]
NHK	0.013 ± 0.002[d,e]	0.02 ± 0.003	0.03 ± 0.006[f]

[a], compared to the 8325-4, $p = 0.006$; [b], compared to the LS-1, $p = 0.0004$;
[c], compared to the 8325-4, $p = 0.002$;
[d], compared to the 8325-4, $p = 0.06$; [e], compared to the LS-1, $p = 0.014$;
[f], compared to the 8325-4, $p = 0.06$.

Data for Figure 4-8a (number of bacteria x10^6 cfu)

S. aureus	Exp1	Exp2	Exp3	Exp4
8325-4	19.7 ± 4.2	22.6 ± 1.6	27.5 ± 2.04	23.0 ± 5.0
LS-1	11.7 ± 1.1	17.5 ± 1.0	21.7 ± 2.2	14.7 ± 1.5
p-value	*0.033*	*0.01*	*0.031*	*0.05*

Data for Figure 4-8b (number of bacteria x10^4 cfu)

S. aureus	Exp1	Exp2	Exp3	Exp4
8325-4	7.3 ± 2.3	4.3 ± 1.7	12.8 ± 2.1	11.7 ± 3.05
LS-1	34.3 ± 5.5	68.3 ± 19.5	121 ± 70	107 ± 30
p-value	*0.001*	*0.005*	*<0.0001*	*0.006*

Data for Figure 4-10 (% of inoculum)

Experiment with UP	*S. aureus* strain			
	8325-4	DU5883	LS-1	LSM
Adhesion	43.70 ± 9.25	14.07 ± 1.12 (*p = 0.005*)	25.92 ± 2.57	11.75 ± 1.94 (*p = 0.002*)
Internalization	0.163 ± 0.05	0.03 ± 0.01 (*p = 0.01*)	0.7 ± 0.1	0.044 ± 0.015 (*p = 0.005*)

p-value compared to the parental strains.

Data for Figures 4-11 and 4-12 (% of inoculum)

Experiment	*S. aureus* strain			
	8325-4	DU5883	LS-1	LSM
Adhesion NHK12P2	10.60 ± 1.95	8.88 ± 3.32	7.20 ± 1.23	3.54 ± 0.68 (*p = 0.01*)
NHK15P2	14.81 ± 5.09	40.86 ± 10.37 (*p = 0.017*)	7.0 ± 1.59	7.33 ± 0.42
NHK22P2	18.05 ± 3.18	27.08 ± 1.8 (*p = 0.01*)	8.95 ± 1.67	8.38 ± 0.87
Internalization NHK12P2	0.02 ± 0.003	0.02 ± 0.002	0.03 ± 0.006	0.01 ± 0.002 (*p = 0.009*)
NHK15P2	0.49 ± 0.057	0.73 ± 0.10 (*p = 0.022*)	0.19 ± 0.017	0.23 ± 0.042
NHK22P2	0.51 ± 0.12	0.52 ± 0.16	0.31 ± 0.08	0.29 ± 0.13

p-value compared to the parental strains.

Data for Figure 4-13 (% of inoculum)

Experiment with H376	*S. aureus*			
	8325-4	DU5883	LS-1	LSM
Adhesion	43.59 ± 7.69	23.25 ± 4.65 (*p = 0.028*)	12.81 ± 0.89	6.48 ± 0.98 (*p = 0.0004*)
Internalization	4.102 ± 0.92	0.008 ± 0.001 (*p = 0.002*)	3.38 ± 1.71	0.003 ± 0.001 (*p = 0.027*)

p-value compared to the parental strains.

Chapter 5

Data for Figure 5-1a

Anti-integrin antibody for	UP	NHK
Adhesion Control	100 ± 6.43	100 ± 12.26
α5 (P1D6)	140.05 ± 12.41 (*p = 0.008*)	145.36 ± 12.76 (*p = 0.01*)
β1 (P4C10 for UP, P5D2 for NHK)	56.63 ± 5.88 (*p = 0.001*)	28.59 ± 4.74 (*p = 0.0007*)
αv (L230)	77.74 ± 12.41 (*p = 0.05*)	104.18 ± 9.28
αvβ6 (10D5)	101.51 ± 10.55	110.42 ± 4.33
β1+αv	54.62 ± 3.92 (*p = 0.0005*)	26.43 ± 11.41 (*p = 0.002*)

p-value compared to 100% value of the control.

Data for Figure 5-1b

Anti-integrin antibody for	UP	NHK
Migration Control (IgG2a)	100 ± 12.4	100 ± 17.74
α5 (P1D6)	20.87 ± 0.69 (*p = 0.0004*)	23.78 ± 6.28 (*p = 0.002*)
β1 (P4C10 for UP, P5D2 for NHK)	17.67 ± 2.25 (*p < 0.0001*)	25.09 ± 2.99 (*p = 0.002*)
αvβ6 (10D5)	95.76 ± 2.27	78.85 ± 28.62
α5+αvβ6	25.19 ± 5.95 (*p = 0.0007*)	Not determined

p-value compared to 100% value of the control.

Data for Figure 5-2

	Internalized *S. aureus* (% of control)		
	UP	NHK	H357
Control	100 ± 16.60	100 ± 14.28	100 ± 26.91
anti-α5 (P1D6)	227 ± 35 (*p = 0.004*)	295 ± 48.6 (*p = 0.003*)	311.76 ± 28.36 (*p = 0.001*)
anti-β1	84.17 ± 8.89	135.71 ± 32.71	Not determined
anti-α5β1 (JBS5)	46 ± 6.7 (*p = 0.0015*)	102.36 ± 5.46	15.0 ± 4.41 (*p = 0.006*)

p-value compared to 100% value of the control.

Data for Figure 5-3a (number of internalized bacteria)

Cell line	*S. aureus* strain		
	NCTC6571	8325-4	LS-1
VB6	6033 ± 115	16000 ± 2000	146700 ± 15300
C1	5367 ± 833	14670 ± 2520	190000 ± 43600

Data for Figure 5-3b (number of internalized bacteria)

Cell line	*S. aureus* strain		
	NCTC6571	8325-4	LS-1
H357	25000 ± 5291	37667 ± 17785	85667 ± 9866
VB6	12000 ± 1000 (*p = 0.014*)	18000 ± 4582	11000 ± 3000 (*p = 0.0002*)
C1	11330 ± 3210 (*p = 0.019*)	16333 ± 6028	18667 ± 5773 (*p = 0.0005*)

p-value compared to the H357

Data for Figure 5-4

Treatment	Internalized *S. aureus* (% of control)	
	UP	NHK
Control in DMEM	100 ± 15.02	100 ± 9.96
10 μg/ml rFnBPBD1-D4	19.72 ± 2.86 (*p = 0.001*)	98.48 ± 10.93
20 μg/ml rFnBPBD1-D4	Not determined	106.52 ± 5.74

p-value compared to the control

Data for Figure 5-5

Treatment	Cell Adhesion (% of control)	
	UP	NHK
Control	100 ± 8.61	100 ± 33.1
1 μg/ml rFnBPBD1-D4	105.9 ± 5.33	105.24 ± 20.18
10 μg/ml rFnBPBD1-D4	95.83 ± 2.55	81.3 ± 27.84
50 μg/ml rFnBPBD1-D4	97.05 ± 2.55	65.06 ± 48.48
100 μg/ml rFnBPBD1-D4	88.35 ± 4.15	66.79 ± 6.9

no significant differences compared to the control

Data for Figure 5-6a (Haptotactic migration)

Coating	Effect of rFnBPBD1-D4 on UP migration		
	Exp1	Exp2	Exp3
Fibronectin (FN)	100 ± 10.49	100 ± 3.3	100 ± 6.06
FN/D1-D4 10μg/ml	85.16 ± 7.24	86.96 ± 14.32	76 ± 19.08
FN/D1-D4 100 μg/ml	67.91 ± 8.37 (*p = 0.014*)	76.81 ± 9.9 (*p = 0.018*)	62.99 ± 17.4 (*p = 0.026*)

Data for Figure 5-6b (wounding assay)

	Effect of rFnBPBD1-D4 on UP migration		
	Exp1	Exp2	Exp3
Fibronectin (FN)	100 ± 2.83	100 ± 11.82	100 ± 4.64
FN/D1-D4 100 μg/ml	63.23 ± 10.09 (*p = 0.038*)	73.21 ± 6.13	61.6 ± 24.47

Data of NHK haptotactic migration in Section 5.3.6

NHK migration		
Fibronectin (FN)	FN/D1-D410μg/ml	FN/D1-D4100 μg/ml
100 ± 33.63	117.45 ± 12.41	86.18 ± 12.0

Chapter 6

Data for Figure 6-3

Experiment		Control	NCTC6571 culture supernatant	
			Exponential phase	Stationary phase
Adhesion	UP	100 ± 12.33	102.82 ± 6.49	110.55 ± 26.21
	NHK	100 ± 7.43	108.82 ± 26.84	103.56 ± 15.01
Migration	UP	100 ± 7.62	92.57 ± 13.6	91.53 ± 3.66
	NHK	100 ± 11.53	85.56 ± 18.91	82.18 ± 13.05

Data for Figure 6-4

Experiment		Control	8325-4 culture supernatant	
			Exponential phase	Stationary phase
Adhesion (n=4)	UP	100 ± 12.81	94.88 ± 8.08	35.48 ± 7.93 (*p = 0.0005*)
	NHK (n=4)	100 ± 8.47	95.36 ± 24.43	45.43 ± 17.43 (*p = 0.008*)
Migration (n=4)	UP	100 ± 8.85	87.06 ± 20.12	7.21 ± 8.58 (*p < 0.001*)
	NHK (n=5)	100 ± 14.07	53.68 ± 19.21 (*p = 0.006*)	0.03 ± 0.06 (*p < 0.001*)

p-value compared to 100% of the control

Data for Figure 6-5

Experiment		Control	LS-1 culture supernatant	
			Exponential phase	Stationary phase
Adhesion	UP	100 ± 12.33	107.49 ± 32.10	67.28 ± 12.47 (*p = 0.045*)
	NHK	100 ± 7.34	98.57 ± 30.38	69.67 ± 26.18
Migration	UP	100 ± 7.23	120.48 ± 28.39	17.77 ± 13.73 (*p = 0.009*)
	NHK	100 ± 10.83	113.86 ± 28.58	8.47 ± 8.53 (*p = 0.003*)

p-value compared to 100% of the control

Data of keratinocyte viability in Section 6.3.5

	Harvested cells (% of control in KGM)	% viable cells	
		Trypan blue	Propidium iodide
NHK : KGM	100%	87.62 ± 4.22	92.26 ± 5.01
8325-4ECS	89.88 ± 8.91	88.81 ± 3.15	94.44 ± 2.24
8325-4SCS	35.45 ± 4.75	79.05 ± 7.80	78.38 ± 6.29
UP : KGM	100%	93.34 ± 2.80	95.74 ± 1.08
8325-4ECS	101.81 ± 14.33	94.14 ± 1.43	97.01 ± 1.07
8325-4SCS	56.61 ± 14.21	91.71 ± 5.24	96.17 ± 1.55

Total cell number and number of viable cells after trypan blue stain were counted using hemocytometer. The number of cells harvested from the control culture in KGM was set as 100%. Viable cells after propidium iodide stain were determined by flow cytometry gating.

Data for Figure 6-8

Treatment	NHK Migration (% of control)	
	8325-4	LS-1
Control in THB	100 ± 16.89	100 ± 9.4
THB/α_2-macroglobulin	97.5 ± 8.12	99.19 ± 6.7
Culture supernatant (CS)	0.97 ± 0.33 ($p < 0.001$)	1.93 ± 1.44 ($p < 0.001$)
CS/α_2-macroglobulin	0.76 ± 0.66 ($p < 0.001$)	5.43 ± 2.41 ($p < 0.001$)
CS/Heat	93.06 ± 17.03	188 ± 32 ($p = 0.01$)

Data for Figure 6-9

Treatment	UP Cell Migration (% of control)	
	8325-4	LS-1
Control in THB	100 ± 6.13	100 ± 6.43
THB/α_2-macroglobulin	95.88 ± 7.46	97.23 ± 6.61
Culture supernatant (CS)	0.75 ± 0.32 ($p < 0.001$)	6.60 ± 2.12 ($p < 0.001$)
CS/α_2-macroglobulin	0.75 ± 0.16 ($p < 0.001$)	5.77 ± 2.3 ($p < 0.001$)
CS/Heat	112.23 ± 4.47 ($p = 0.049$)	127.21 ± 8.06 ($p = 0.01$)

Chapter 7

Data for Figure 7-6a (% cell adhesion of UP cells)

	2 h		24 h	
	8325-4	DU1090	8325-4	DU1090
0.01%	90 ± 2.8	127 ± 5.2	98.15 ± 3.28	97.23 ± 0.65
0.1%	102.75 ± 10.17	110.65 ± 3.85	97.46 ± 2.26	100.46 ± 10.44
1%	21.55 ± 3.34 ($p < 0.001$)	78.31 ± 2.71	26.19 ± 5.85 ($p < 0.001$)	88.97 ± 4.48
5%	8.64 ± 2.7	15.74 ± 0.9	0 ($p < 0.001$)	53.88 ± 3.51
25%	3.68 ± 3.13	0	0	0

Data for Figure 7-6b (% cell adhesion of NHK)

	2 h		24 h	
	8325-4	DU1090	8325-4	DU1090
0.01%	100	100	100	100
0.1%	55.09 ± 11.87	72.67 ± 8.54	75.37 ± 4.9	79.57 ± 10.99
1%	14.31 ± 1.91 ($p < 0.01$)	68.11 ± 4.13	45.67 ± 12.13 ($p < 0.05$)	76.27 ± 12.16
5%	6.55 ± 0.2	14.66 ± 0.2	16.85 ± 1.92 ($p < 0.01$)	69.87 ± 0.35
25%	2.01 ± 1.92	0.6 ± 0.38	0.87 ± 0.28	4.89 ± 0.79

Data for Figure 7-8a

	% PI positive UP cells at 24 h		
	THB	8325-4	DU1090
0.01%	2.79 ± 0.44	3.09 ± 0.6	3.25 ± 0.09
0.1%	3.11 ± 0.23	3.09 ± 0.41	4.4 ± 2.47
1%	3.18 ± 0.27	3.61 ± 0.18	5.78 ± 5.56
5%	3.02 ± 0.39	3.38 ± 0.48	30.67 ± 2.16
25%	2.75 ± 0.3	7.96 ± 1.48	95.51 ± 0.59

Data for Figure 7-8b

	% PI positive NHK at 24 h		
	THB	8325-4	DU1090
0.01%	5.93 ± 0.03	6.21 ± 0.06	5.66 ± 0.25
0.1%	6.00 ± 0.25	9.80 ± 0.51	5.84 ± 0.19
1%	5.88 ± 0.07	14.10 ± 1.36	8.28 ± 0.01
5%	6.05 ± 0.03	25.21 ± 0.59	37.91 ± 0.61
25%	5.88 ± 0.04	48.45 ± 3.56	81.04 ± 0.85

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