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Adherence of Skin Bacteria to Human Epithelial Cells

SANDRA ROMERO-STEINER,[1]† THOMAS WITEK,[1] AND EDWARD BALISH[1,2]*

Departments of Medical Microbiology[1] and Surgery,[2] University of Wisconsin Medical School, 1300 University Avenue, Madison, Wisconsin 53706

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Aerobic and anaerobic bacteria isolated from human axillae were tested for their capacity to adhere to buccal epithelial cells, immortalized human epithelial (HEp-2) cells, and undifferentiated and differentiated human epithelial cells. In general, both aerobic and anaerobic diphtheroids adhered better to differentiated human epithelial cells than to HEp-2 and undifferentiated human epithelial cells ($P < 0.05$). Mannose, galactose, fucose, *N*-acetyl-D-glucosamine, and fibronectin were also assayed for their capacity to inhibit the adherence of diphtheroids to human epithelial cells. A great deal of variability was observed in the capacity of the latter compounds to inhibit the attachment of aerobic diphtheroids to undifferentiated and differentiated epithelial cells. Overall, mannose appeared to be best at inhibiting the adherence of the aerobic diphtheroids to undifferentiated human epithelial cells. Galactose, fucose, *N*-acetyl-D-glucosamine, and fibronectin showed a greater capacity to inhibit attachment of aerobic diphtheroids to differentiated than to undifferentiated human epithelial cells. The inhibition of adherence to differentiated human epithelial cells varied with the microorganism and the compound tested; however, the highest and most consistent inhibition of adherence (76.1 to 88.6%) was observed with a 5% solution of *N*-acetyl-D-glucosamine. The in vitro adherence and adherence inhibition assays presented here demonstrate that a number of adhesins and receptors are involved in the adherence of skin bacteria to human epithelial cells and receptors on human epithelial cells are apparently altered during differentiation.

The bacterial flora of skin consists primarily of staphylococci, micrococci, corynebacteria, and propionibacteria (12). The distribution of these skin bacteria in normal healthy individuals has been detailed previously (12, 13). The number and kind of bacteria present on skin vary with the site cultured, sampling and culture methods utilized, availability of moisture, nutrients, pH, and body temperature (2, 12). The axilla is one of the body sites that supports a large population of bacteria and is an ideal area in which to study skin microflora. The presence of eccrine and apocrine sweat gland products and moisture in the axilla provide a diversity of nutrients that supports the growth of a variety of nonlipophilic and lipophilic microorganisms (11, 13). Lipophilic corynebacteria are found in high numbers in the axilla (2, 9, 16). Despite the fact that many studies have quantitated and classified these bacteria, very little is known about the chemical basis for adherence of these diphtheroids to epithelial cells (9, 13, 16, 23). In the present study, samples from the axillae of 10 human volunteers were cultured and the bacterial isolates were identified and quantitated. From these isolates, aerobic and anaerobic diphtheroids were tested for their capacity to adhere to buccal epithelial cells (BEC), HEp-2 cells, and undifferentiated and differentiated human epithelial (HE) cells. HE cells in vitro can be induced to differentiate into keratinocytes (20, 22, 24). In addition to the adherence assays, several compounds were tested for their capacity to inhibit adherence of diphtheroids to the HE cells used in the in vitro adherence assays.

MATERIALS AND METHODS

Microorganisms. Skin microorganisms were isolated from the axillae of 10 healthy human volunteers (males and females). *Corynebacterium diphtheriae*, *Corynebacterium* group JK, and *Propionibacterium acnes* and *Escherichia coli* 1677 were obtained, respectively, from the culture collection at the State Laboratory of Hygiene, Madison, Wis., and from Walter J. Hopkins, Department of Surgery, University of Wisconsin, Madison. These four bacteria were included as controls for the adherence experiments. Microorganisms were maintained by subculture on blood agar plates prepared with Trypticase soy agar base (BBL Microbiology Systems, Cockeysville, Md.), 0.5% yeast extract (Difco Laboratories, Detroit, Mich.), 5% defibrinated sheep blood (GIBCO Diagnostics, Madison, Wis.), and 0.5% Tween 80 (Sigma Chemical Co., St. Louis, Mo.) as a supplement for lipophilic microorganisms. Stock cultures were frozen at −70°C in semisolid Trypticase soy agar for aerobes and chopped-meat–carbohydrate broth for anaerobes. For in vitro adherence assays, the lipophilic corynebacteria were serially subcultured three times for 48-h periods in tryptose broth (Difco) with 0.5% Tween 80.

Isolation and identification. Isolation of microorganisms from the axillae was performed by addition of 1 ml of a 0.1% Triton X-100 solution onto a sterile glass cylinder placed firmly on the skin in the axilla (total area, 3.8 cm^2). The fluid was stirred vigorously for 1 min with a Teflon rod (policeman) and collected (12, 25). The latter procedure was repeated and the two samples were pooled. To help in the recovery of anaerobes, O_2-free CO_2 was applied over the collected samples and the tubes were then capped tightly and cooled to 4°C. All samples were processed within 8 h of collection. Volunteers were asked not to use deodorants and to wash with only a mild soap (Ivory) for 8 days prior to sampling of axillae. Individual samples were serially diluted in 10-fold dilutions and plated onto nonprereduced and prereduced blood agar plates. Aerobic and anaerobic incubation conditions were maintained for 3 to 5 days at 37°C, at which time final bacterial counts of both aerobes and anaerobes were made. Axillary samples were processed inside an anaerobic glove box (Coy Laboratory Products, Ann Arbor,

* Corresponding author.
† Present address: Laboratory for Microbial and Biochemical Sciences, Georgia State University, Atlanta, GA 30303.

Mich.). Aerobic microorganisms were identified by biochemical characterization by established procedures, as described in the *Manual of Clinical Microbiology* (6). Anaerobic microorganisms were identified by procedures established by the Virginia Polytechnic Institute (5) and by gas chromatography (DODECA, model-700A). All microorganisms were tested for lipophilicity by subculture on blood agar plates with 0.5% Tween 80 and incubation under aerobic or anaerobic conditions at 37°C for 48 h. Microorganisms that demonstrated enhanced growth or pigmentation on this lipid-supplemented growth medium were considered lipophilic.

Epithelial cells. BEC and two cell lines, HE cells (primary culture from normal human epidermal keratinocytes, EPIPACK cells, from Clonetics Corp., San Diego, Calif.) and HEp-2 cells (immortalized HE cells, line 197), were used to assess the adherence of the axillary microorganisms. BEC were obtained by scrapping the oropharynx of a healthy human volunteer with a wooden tongue depressor. Cells were suspended in phosphate-buffered saline and washed three times by differential centrifugation (500 × *g*) with a vigorous agitation between washes (19). Differentiation of HE cells was induced by addition of calcium chloride ($CaCl_2$) to a concentration of 2 mmol/liter (22). Differentiation was assessed by mound formation (three to five layers of cells) during tissue culture and by a modified Ayoub-Shklar stain for differentiated epithelial cells (3). HEp-2 cells were kindly donated by the tissue culture section of the State Laboratory of Hygiene, Madison, Wis. HE cells were grown in keratinocyte growth medium (serum-free; Clonetics Corp.), while HEp-2 cells were grown in basal medium Eagle (GIBCO) supplemented with 10% fetal calf serum (GIBCO). Cell lines were cultured in an atmosphere of 5% CO_2 at 37°C. Cell lines were subcultured at the following intervals: 4 days for HEp-2 cells, 8 days for undifferentiated HE cells, and 8 to 16 days for differentiated HE cells. Both cell lines were harvested by addition of a trypsin (0.25 g/liter)–EDTA (0.1 g/liter) solution as described previously (15) with the following modifications: HE cells were washed with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer prior to the trypsinization, and an antitrypsin solution (Clonetics Corp.) was added to neutralize the enzymatic reaction. Cells were allowed to recover from the trypsinization procedure for 6 to 8 h in growth medium under a 5% CO_2 atmosphere at 37°C. Harvested cells were washed three times by centrifugation (3 min, 1,500 × *g*) with 0.067 M phosphate-buffered saline (pH 7.2) and suspended in Dulbecco phosphate-buffered saline (10×; GIBCO) to a final concentration of 0.5×10^6 to 1.0×10^6 cells per ml. Suspensions were standardized in a hemacytometer (American Optical Corp., Buffalo, N.Y.) prior to use.

In vitro bacterial adherence assays. Individual bacterial suspensions, 50 μl each (1.5×10^8 bacteria per ml for anaerobes and 3.0×10^8 bacteria per ml for aerobes), were added to 50 μl of cell suspension (0.5×10^6 to 1.0×10^6 cells per ml) and incubated for 30 min with rocking (Orbitron rotator; Boekel Industries, Inc., Philadelphia, Pa.) at room temperature (18). A 25-μl inoculum was vacuum filtered onto a polycarbonate filter (pore size, 8.0 μm; Nuclepore Corp., Pleasanton, Calif.). The cells on the filter were washed three times with sterile distilled water to remove nonadherent bacteria. After fixation with 95% ethanol, the cells with attached bacteria were Gram stained on the filter as described previously (21). The number of bacteria attaching to 20 epithelial cells was counted by using a light microscope (Nikon OPTIPHOT) with a total magnification of ×1,000. Experiments were run in duplicate, and three individual blind readings were made on each sample.

Inhibition of bacterial adherence. The following compounds were assayed for their effects on the adherence of skin microorganisms to epithelial cells: human fibronectin (FN; 50 μg/ml) and 17-mg/ml final concentrations of D-(+)-fucose, D-(+)-galactose, D-(+)-mannose, and *N*-acetyl-D-glucosamine (NAGA). These compounds were purchased from Sigma Chemical Co. In the adherence inhibition assays, 50 μl of the compound to be tested was added to the HE cells in vitro 5 min before the bacterial suspensions were added.

Statistical analysis. Student's *t* test for determining significant differences between pairs of data was used to compare the results from the adherence and inhibition of adherence assays. A Minitab program for IBM computers was used, and the *P* value was set at 0.05 before the experiments were run.

RESULTS

Microorganisms. Forty-nine aerobic and anaerobic microorganisms were isolated from the axillae of 10 healthy male and female human volunteers (Table 1). As a group, the staphylococci were most frequently isolated, and *Staphylococcus hominis* was isolated from 8 of the 10 volunteers. Lipophilic aerobes and anaerobes were isolated from 7 of 10 subjects (Table 1). The number of bacteria isolated varied widely: 5.0×10^2 to 2.9×10^6 CFU/ml per 3.8 cm^2 for lipophilic aerobes and 2.0×10^2 to 5.0×10^6 CFU/ml per 3.8 cm^2 for lipophilic anaerobes. All anaerobes isolated from the 10 subjects were lipophilic. Nonlipophilic aerobes predominated in all of the subjects, and a wide concentration range (4.0×10^1 to 6.9×10^7 CFU/ml per 3.8 cm^2) was evident (Table 1).

Adherence assays. Sixteen aerobic and anaerobic diphtheroids, one *Streptococcus* sp., and one *Lactobacillus* sp. were tested for adherence to BEC, HEp-2 cells, and undifferentiated and differentiated HE cells. In addition to these microorganisms, a *Corynebacterium* group JK, *C. diphtheriae*, *E. coli* 1677, and *P. acnes* were included as controls. The average number (based on six readings of two assays) of aerobic diphtheroids adhering to 20 epithelial cells is shown in Table 2. With the exception of *C. minutissimum* and *C. diphtheriae* (SLH), the aerobic diphtheroids showed a better capacity to adhere to differentiated HE cells than to the other epithelial cells used in this study. *Corynebacterium* group G-2 attached well to all epithelial cells used, but attached in very large numbers to differentiated HE cells. Table 3 shows the results of the in vitro adherence of anaerobic skin isolates and a *P. acnes* control to the epithelial cells. The propionibacteria also exhibited a significant increase in their capacity to attach to differentiated HE cells. In general, both aerobic and anaerobic diphtheroids (Tables 2 and 3) adhered better to differentiated HE cells than to BEC, HEp-2 cells, and undifferentiated HE cells ($P < 0.05$).

Adherence inhibition studies. Mannose, galactose, fucose, NAGA, and FN were assayed for their capacity to interfere with the adherence of aerobic diphtheroids to undifferentiated and differentiated HE cells (Table 4). All of the compounds had the capacity to inhibit attachment of skin bacteria to differentiated or undifferentiated HE cells. Overall, mannose appeared to have the best capacity to inhibit bacterial adherence to undifferentiated cells (87.5 to 99.4% inhibition); however, mannose inhibition of adherence to differentiated cells (53.9 to 90.3%) varied with the microor-

TABLE 1. Bacteria isolated from human axillae

Bacterial strains	Frequency of isolation from 10 subjects	Range observed (CFU/ml per 3.8 cm^2)
Aerobic, nonlipophilic		
Staphylococcus hominis	8	1.9×10^3–5.0×10^6
S. sciuri	1	1.7×10^4
S. haemolyticus	5	1.2×10^2–9.0×10^5
S. warneri	3	1.7×10^4–6.9×10^7
S. epidermidis	5	3.2×10^2–3.6×10^4
S. caprae	1	8.5×10^5
S. auricularis	1	1.6×10^5
S. capitis	1	1.3×10^7
Micrococcus luteus	3	8.0×10^1–5.3×10^6
M. sedentarius	1	6.4×10^3
M. varians	1	4.0×10^1
Flavobacterium odorans	1	1.5×10^5
Aerobic, lipophilic		
Corynebacterium minutissimum	2	2.4×10^3–1.8×10^4
C. pseudotuberculosis	1	2.9×10^6
Corynebacterium group F-1	1	6.0×10^2
Corynebacterium group JK	1	1.7×10^6
Corynebacterium group G-2	1	8.0×10^4
C. xerosis	1	5.0×10^2
Corynebacterium group C	1	4.4×10^3
Anaerobic, lipophilic		
Propionibacterium acnes serotype 1	3	7.0×10^2–1.3×10^5
P. acnes serotype 2	4	2.0×10^2–5.0×10^6
P. thoenii	1	3.3×10^4
Lactobacillus sp.	1	4.4×10^6
Streptococcus intermedius	1	1.6×10^4

ganism tested and was not consistently high with all of the isolates tested.

With the exception of a poor capacity to inhibit attachment of *C. pseudotuberculosis* (isolate 1) and *Corynebacterium* group JK, galactose and NAGA were able to inhibit attachment of aerobic skin diphtheroids to differentiated epithelial cells better (73.3 to 95.8% inhibition; Table 4) than fucose and FN (but not always). In general, fucose, NAGA, and FN were consistently able to inhibit attachment of aerobic skin diphtheroids to both differentiated and undifferentiated HE cells; however, inhibition of adherence was more evident with differentiated than undifferentiated HE cells.

DISCUSSION

Gram-positive bacteria are the predominant microflora found in the axillary area of humans. The spectrum of microorganisms we isolated is in good agreement with previous reports of skin microflora in the human axillae (8, 9, 12). Of 49 isolates, 18 showed enhanced growth and pigment production when lipid supplement (0.5% Tween 80) was added to the medium. It is thought that increased concentration of lipid from axillary apocrine sweat supports the growth of these lipophilic skin bacteria (11, 13). We isolated a variety of species belonging to the *Staphylococcus* and *Corynebacterium* genera (Table 1). The majority of axillary isolates were aerobic, and only one-fifth of the isolates were anaerobic. *Flavobacterium odorans* was the only gram-negative bacterium isolated.

HE cells were used to study the adherence of the lipophilic isolates. In general, the aerobic diphtheroids attached better

TABLE 2. Adherence of aerobic skin bacteria to HE cells[a]

Bacterial strains	Avg no. of bacteria attaching to 20 cells ± SE (n = 6)			
	BEC	HEp-2 cells	HE cells[b]	
			Undifferentiated	Differentiated
C. minutissimum				
Isolate 1	93 ± 18	211 ± 16	193 ± 11	190 ± 5
Isolate 2	95 ± 23	169 ± 11	128 ± 12	498 ± 11
C. pseudotuberculosis				
Isolate 1	78 ± 8	135 ± 3	172 ± 7	234 ± 9
Isolate 2	133 ± 5	126 ± 6	158 ± 9	210 ± 14
Corynebacterium group F-1	97 ± 12	187 ± 10	183 ± 10	434 ± 22
Corynebacterium group G-2	176 ± 8	166 ± 12	253 ± 26	>500
C. xerosis	70 ± 10	148 ± 10	201 ± 15	266 ± 16
Corynebacterium group C	182 ± 9	136 ± 13	169 ± 7	324 ± 6
Corynebacterium group JK	129 ± 7	187 ± 11	191 ± 7	316 ± 28
Corynebacterium group JK (SLH)	134 ± 6	195 ± 13	182 ± 11	201 ± 24
C. diphtheriae (SLH)	111 ± 9	146 ± 7	94 ± 9	115 ± 6
E. coli 1677[c]	309 ± 10	346 ± 51	271 ± 16	267 ± 14

[a] Average cell sizes: BEC, 49 μm; HEp-2 cells, 14.3 μm; HE undifferentiated, 12.2 μm; HE differentiated, 14.3 μm.
[b] Differentiation of EPIPACK HE cells was induced by addition of calcium chloride (final concentration, 2 mmol/liter [22]) to the growth medium.
[c] Used as a gram-negative control for adherence studies.

TABLE 3. Adherence of anaerobic skin bacteria to HE cells[a]

Bacterial strains	Avg no. of bacteria attaching to 20 cells ± SE (n = 6)			
	BEC	HEp-2 cells	HE cells[b]	
			Undifferentiated	Differentiated
P. acnes serotype I				
Isolate 1	13 ± 4	9 ± 1	3 ± 1	109 ± 12
Isolate 2	5 ± 1	8 ± 1	7 ± 2	99 ± 9
Isolate 3	24 ± 4	7 ± 2	28 ± 12	112 ± 11
P. acnes serotype II				
Isolate 1	21 ± 4	11 ± 2	2 ± 1	72 ± 2
Isolate 2	30 ± 5	13 ± 4	9 ± 3	75 ± 11
Isolate 3	13 ± 3	5 ± 1	15 ± 3	108 ± 7
Isolate 4	8 ± 3	10 ± 1	11 ± 1	83 ± 5
P. thoenii	4 ± 1	15 ± 3	2 ± 1	43 ± 2
Lactobacillus sp.	4 ± 1	8 ± 2	164 ± 14	39 ± 7
Streptococcus intermedius	5 ± 1	9 ± 2	39 ± 11	38 ± 3
P. acnes (SLH), serotype I	3 ± 1	7 ± 1	39 ± 11	129 ± 15

[a] Average cell sizes: BEC, 49 μm; HEp-2 cells, 14.3 μm; HE undifferentiated, 12.2 μm; HE differentiated, 14.3 μm.
[b] Differentiation induced by addition of calcium chloride to a final concentration of 2 mmol/liter (22).

TABLE 4. Inhibition of adherence of aerobic bacteria to HE cells

Bacterial strain	HE cells	% Inhibition of bacterial attachment by:					No inhibition (controls; no. of bacteria per 20 epithelial cells)[a]
		Mannose[b]	Galactose[b]	Fucose[b]	NAGA[b]	FN[c]	
C. pseudotuberculosis isolate 1	Undifferentiated	94.20	−4.60	−8.70	43.60	63.40	172 ± 7
	Differentiated[d]	53.90	15.80	35.50	76.10	63.70	234 ± 9
Corynebacterium group F-1	Undifferentiated	95.60	26.80	27.30	59.00	45.90	183 ± 10
	Differentiated	78.30	88.30	89.20	86.90	83.90	434 ± 22
Corynebacterium group JK	Undifferentiated	92.20	8.40	25.10	53.90	34.60	191 ± 7
	Differentiated	69.30	77.20	54.40	81.70	88.90	316 ± 28
C. minutissimum isolate 2	Undifferentiated	87.50	24.20	57.00	65.60	65.60	128 ± 12
	Differentiated	90.30	95.80	84.50	88.60	95.00	498 ± 11
Corynebacterium group G-2	Undifferentiated	88.50	60.50	44.30	59.30	53.40	253 ± 26
	Differentiated	78.00	84.40	77.80	87.80	73.20	500
C. xerosis	Undifferentiated	96.50	60.70	79.60	73.10	63.20	201 ± 15
	Differentiated	88.40	73.30	77.80	82.00	72.90	266 ± 16
Corynebacterium group C	Undifferentiated	99.40	78.70	49.70	62.10	36.40	169 ± 7
	Differentiated	71.00	85.80	63.00	80.60	76.90	324 ± 6

[a] Average ± standard error; data represent six readings of two adherence assays.
[b] 50 μl of a 5% (17-mg/ml) sugar or NAGA solution.
[c] 50-μg/ml solution of FN.
[d] Differentiation of HE cells induced by addition of calcium chloride to a final concentration of 2 mmol/liter (22).

to all cell types (70 to >500 bacteria per 20 epithelial cells) than the anaerobic diphtheroids (2 to 112 bacteria per 20 epithelial cells). *E. coli*, *C. diphtheriae*, *Lactobacillus* sp., and *Streptococcus intermedius* demonstrated a comparable capacity to attach to all three epithelial cell types used. Both aerobic and anaerobic diphtheroids demonstrated an increased capacity to adhere to differentiated HE cells. These results were expected since, of the cells used, the differentiated HE cells most closely resemble the surface epithelial cells found in the epidermis. The adherence of the aerobic diphtheroids to all cell types was approximately 10 times greater than the adherence of the anaerobic diphtheroids. Variability in the adherence capacity of different isolates of aerobic and anaerobic diphtheroids was evident. For example, *C. minutissimum* (isolate 2), *Corynebacterium* group G-2, and *P. acnes* serotype 1 (isolate 3) demonstrated a greater capacity to adhere to differentiated HE cells than the other aerobic and anaerobic isolates.

It has been reported that the cell wall of bacteria in the genus *Corynebacterium* contains NAGA and *meso*-diaminopimelic acid as well as arabinose, galactose, and mannose (13). Galactose, mannose, and NAGA were tested for their capacity to inhibit adherence of bacteria to epithelial cells. Although the adherence of different bacterial isolates was inhibited to various degrees by the compounds tested, mannose gave the best inhibition of bacterial adherence to undifferentiated HE cells, whereas galactose, FN, and NAGA showed a good capacity to inhibit adherence to differentiated HE cells. These adherence studies indicate that a mannose-binding adhesin on the cell wall of diphtheroids may be implicated in their adherence to undifferentiated HE cells. Similarly, mannose and galactose receptors may be involved in bacterial adherence to differentiated HE cells. FN may also play a role in attachment of diphtheroids to undifferentiated and differentiated HE cells. Our results clearly indicate that several factors mediate the adherence of these skin microorganisms to epithelial cells. Most of the previous work on bacterial adherence has focused on the attachment of gram-negative rather than gram-positive bacteria (1, 4, 7, 10, 14, 19). The in vitro adherence and adherence inhibition assays presented herein demonstrate that bacterial adhesins and receptors on epithelial cells may play an important role in skin ecology and may explain the predominance and persistance of certain groups of bacteria in these ecosystems.

The use of epithelial cells derived from human skin provides a convenient in vitro model for the study of adherence mechanisms of skin microorganisms. There have been several attempts to perform bacterial adherence studies directly on human skin; however, the methodology substantially modifies the skin environment and thus its indigenous microflora (12, 16). In addition, other normal flora microorganisms may interfere with such in situ studies, and certain skin cleansing agents could modify adhesins and receptors. The model presented herein is sensitive, specific, quantitative, and versatile and should be useful for future in vitro studies on adherence of skin microflora to HE cells.

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