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Epithelial Cell Polarity Affects Susceptibility to *Pseudomonas aeruginosa* Invasion and Cytotoxicity

SUZANNE M. J. FLEISZIG,[1]* DAVID J. EVANS,[1] NINA DO,[1] VICKY VALLAS,[1] SUSAN SHIN,[1] AND KEITH E. MOSTOV[2]

School of Optometry, University of California, Berkeley, Berkeley, California 94720,[1] and Department of Anatomy, School of Medicine, University of California, San Francisco, San Francisco, California 94143[2]

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Intact tissues are relatively resistant to *Pseudomonas aeruginosa*-induced disease, and injury predisposes tissue to infection. Intact epithelia contain polarized cells that have distinct apical and basolateral membranes with unique lipids and proteins. In this study, the role of cell polarity in epithelial cell susceptibility to *P. aeruginosa* virulence mechanisms was tested. Madin-Darby canine kidney (MDCK) cells, human corneal epithelial cells, and primary cultures of two different types of airway epithelial cells were grown on Transwell filters or in plastic tissue culture wells. *P. aeruginosa* invasion of cells was quantified by gentamicin survival assays with two isolates that invade epithelial cells (6294 and PAO1). Cytotoxic activity was assessed by trypan blue exclusion assays with two cytotoxic strains (6206 and PA103). Basolateral surfaces of cells were exposed by one of two methods: EGTA pretreatment of epithelial cells or growth of cells in low-calcium medium. Both methods of exposing basolateral membranes increased epithelial cell susceptibility to *P. aeruginosa* invasion and cytotoxicity. Migrating cells were also found to be more susceptible to *P. aeruginosa* invasion than confluent monolayers that had established membrane polarity. Monolayers of MDCK cells that had been selected for resistance to killing by concanavalin A were resistant to both cytotoxicity and invasion by *P. aeruginosa* because they were more efficiently polarized for their susceptibility to *P. aeruginosa* virulence factors than regular MDCK cells and not because they were defective in glycosylation. These results suggest that there are factors on the basolateral surfaces of epithelial cells that promote interaction with *P. aeruginosa* or that there are inhibitory factors on the apical cell surface. Thus, cell polarity of intact epithelia is likely to contribute to defense against *P. aeruginosa* infection.

Pseudomonas aeruginosa is an opportunistic pathogen of humans that can infect a wide variety of epithelial tissues (31). We have found that clinical isolates of *P. aeruginosa* are of at least two different types (12). One type can invade epithelial cells in vitro (6, 11, 26) and during infection in the eye (9) and the lung (13, 26). Other *P. aeruginosa* strains are cytotoxic for epithelial cells (1, 12, 20). Since healthy tissues are normally resistant to infection with *P. aeruginosa*, some form of compromise of host defenses must be necessary for these bacteria to cause tissue disease.

Epithelial cells throughout the body are polarized. Polarized epithelial cells are characterized by apical and basolateral domains which differ in the composition of their membrane constituents. Tight junctions segregate these domains, and there is targeted delivery and retention of constituents either to the apical or the basolateral surface of the cell (33). Receptors for various ligands are among the proteins which are preferentially expressed at either surface of an epithelial cell.

In intact healthy tissues only apical membranes are exposed to the environment. When injured, epithelial tissues may become susceptible to *P. aeruginosa* infection (28). Since injury exposes the basolateral sides of cells, we hypothesized that cell polarity in healthy tissues may play a significant role in resistance to infection with this bacterium. Basolateral cell membranes have been shown to be more susceptible to *Listeria monocytogenes* and *Shigella flexneri* invasion than apical cell surfaces (15, 25). The receptor for *L. monocytogenes* invasion was recently shown to be E-cadherin, a factor found on basolateral epithelial cell surfaces that is involved in the establishment of cell-to-cell junctions (23).

Madin-Darby canine kidney (MDCK) cells, corneal epithelial cells, and primary cultures of two different types of airway epithelial cells were used to test *P. aeruginosa* interactions with cells in which basolateral membranes were exposed by various methods. The results showed that for all four cell types there was significantly greater susceptibility to *P. aeruginosa* invasion and cytotoxicity when bacteria were added to cells that had lost their polarity.

MATERIALS AND METHODS

Bacteria. Four nonmucoid isolates of *P. aeruginosa* were tested (serogroup O2, PAO1; serogroup O6, 6294; and serogroup O11, 6206 and PA103). Isolates PAO1 and 6294 invade cells without inducing cytotoxicity (13). Strains 6026 and PA103 are cytotoxic (13). Bacteria were grown overnight at 37°C on a Trypticase soy agar plate (8) and were resuspended in tissue culture medium at various concentrations determined by spectrophotometry (optical density at 650 nm) and confirmed by viable counts.

Preparation of cell cultures. Corneal epithelial cells and the two different types of airway cells were selected for use in these experiments because the cornea and the lung are two of the most common sites for *P. aeruginosa*-induced disease. We chose to support the data with experiments performed with MDCK type II (hereafter referred to as MDCK) cells because they form polarized monolayers and they have been used extensively as a model system for studying the manner in which epithelial cells form and maintain polarity (27, 33). Furthermore, the effects of the various manipulations used in this study are well understood for MDCK cells (17, 30, 32). MDCK cells that had been selected for their resistance to killing by the lectin concanavalin A ($ConA^r$ cells) were also used (22). $ConA^r$ cells to be used for seeding onto filters or into plastic wells were grown in the presence of 50 μg of concanavalin A/ml (EY Laboratories, Inc., San Mateo, Calif.) on 10-cm-diameter tissue culture dishes (Falcon, Lincoln Park, N.J.) to maintain the stability of their resistance to this lectin. MDCK cells were found to be killed by this concentration of concanavalin A (data not shown). Concanavalin

* Corresponding author. Mailing address: School of Optometry, University of California, Berkeley, CA 94720-2020. Phone: (510) 643-0990. Fax: (510) 643-5109. E-mail: fleiszig@socrates.berkeley.edu.

TABLE 1. EGTA pretreatment of epithelial cells significantly increased their susceptibility to *P. aeruginosa* invasion

Cell type[a]	EGTA treatment		Invasion (CFU)[b]		Association (CFU)[c]		TER (Ω/cm^2)[d]	
	Time (min)	Concn	Control	EGTA treated	Control	EGTA treated	Control	EGTA treated
MDCK (filter grown)	60	30 mM	$(4.9 \pm 0.6) \times 10^3$	$(5.3 \pm 0.7) \times 10^4$	$(7.3 \pm 7.4) \times 10^4$	$(3.3 \pm 0.4) \times 10^5$	200 ± 5	115 ± 1
MDCK (plastic grown)	30	30 mM	$(3.8 \pm 0.3) \times 10^3$	$(3.2 \pm 0.2) \times 10^4$	NA[e]	NA	NA	NA
HCE	90	30 mM	$(4.7 \pm 0.2) \times 10^3$	$(6.1 \pm 0.3) \times 10^4$	$(7.9 \pm 0.6) \times 10^4$	$(1.8 \pm 0.1) \times 10^6$	339 ± 10	111 ± 2
Bovine tracheal epithelium	30	100 μM	530 ± 210	$(5.3 \pm 0.3) \times 10^4$	$(2.5 \pm 0.7) \times 10^5$	$(3.8 \pm 0.7) \times 10^6$	734 ± 48	207 ± 13
Human nasal epithelium	30	500 μM	3 ± 1	$(2.8 \pm 0.5) \times 10^3$	$(1.5 \pm 0.3) \times 10^3$	$(4.0 \pm 1.7) \times 10^5$	$1{,}637 \pm 51$	265 ± 6

[a] The inoculum was 1.5×10^5 CFU for MDCK cells and 1.5×10^6 for all other cell types. Strain 6294 was used for MDCK and HCE cells; strain PAO1 was used for experiments with respiratory cells. Numbers indicate means ± standard errors of the means ($P < 0.01$) for all cell types.
[b] Invasion was measured as the number of bacteria associated with cells that survived 2 h of treatment with 200 μg of gentamicin/ml.
[c] Association was measured as the total number of bacteria recovered from cells after washing to remove nonadherent bacteria.
[d] Measured with an EVOM meter.
[e] NA, not applicable. Association was not measured for cells grown on plastic (see Materials and Methods).

A was not included in the growth medium after seeding ConA^r cells in plastic wells or on filters to be used for bacterial interaction assays.

MDCK or ConA^r cells were plated onto 0.4-μm-pore-size, 12-mm-diameter Transwell filters (Costar Corp., Cambridge, Mass.) or 15-mm-diameter plastic tissue culture wells (Falcon) (5). Cells were plated at a density of 4×10^5 cells/cm^2 and maintained at 37°C in modified Eagle's medium (MEM) with Earle's balanced salt solution (Cellgro; Mediatech, Herndon, Va.) containing 10% fetal bovine serum (Hyclone Laboratories Inc., Logan, Utah). The medium was changed each of the two days following plating, and the cells were used on the third day.

Similar methods were used to grow human corneal epithelial (HCE) cells (2) except that they were grown in SHEM for 7 days as previously described (12).

Primary cultures of bovine tracheal cells and primary cultures of human nasal cells were grown on collagen-coated Transwell filters. Techniques for culturing these cell types, which have been shown to have properties similar to original tissue with respect to their ultrastructure and ion transport, have been described previously (36). Briefly, strips of tracheal epithelium were pulled away from underlying tissues, and epithelial cells were isolated by overnight digestion (4°C) with 0.05% (wt/vol) protease (type XIV; Sigma, St. Louis, Mo.) in phosphate-buffered saline. The next day, the protease solution was removed and replaced with the cell culture medium consisting of equal volumes of Dulbecco's modified Eagle's medium and Ham's F-12 medium and containing fetal calf serum (5%, vol/vol), penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (50 μg/ml), amphotericin B (2.5 μg/ml), transferrin (5 μg/ml), hydrocortisone (0.36 μg/ml), endothelial cell growth supplement (7.5 μg/ml), insulin (10 μg/ml), epidermal growth factor (25 ng/ml), and triiodothyronine (20 ng/ml). Epithelial cells were dispersed from tissue strips by vigorous shaking (5 min), pelleted by centrifugation ($200 \times g$, 10 min), and resuspended into the cell culture medium. Epithelial cells were plated at a cell density of 10^6 cells/cm^2 onto 12-mm-diameter, 0.4-μm-pore-size Transwell filters (Costar) coated with a thin film of human placental collagen (20 μg/ml). Cells were grown at an air-liquid interface (no medium was added to the mucosal surface) and fed every alternate day with the culture medium described above. Confluent, polarized sheets of airway surface epithelium developed after approximately 5 days. For experiments described in this study, epithelial sheets were between 8 and 17 days old.

Transepithelial resistance (TER) of cells grown on filters was monitored with an EVOM meter (World Precision Instruments Inc., Sarasota, Fla.). As was expected for all cell types, TER increased when cells became confluent, indicating that tight junctions had formed; when tight junctions were disrupted, TER decreased to near the baseline levels measured for control filters with no cells.

Disruption of tight junctions. Two different methods were used to produce cells in which basolateral membrane constituents were exposed to the apical cell surface; both methods involved disrupting tight junctions by depleting calcium from the medium (21).

(i) EGTA treatment. EGTA (Sigma) was prepared in Hanks' balanced salt solution (Ca^{2+}- and Mg^{2+}-free) (University of California Cell Culture Facility, San Francisco). EGTA pretreatment upsets cell polarity by chelating calcium and thus disrupting existing tight junctions (21). Cells were treated with EGTA and then washed to remove EGTA before the addition of bacteria. The pretreatment time and EGTA concentration necessary to disrupt tight junctions were found to vary with the cell type tested (Table 1). Disruption of tight junctions was assessed by one of two methods. For cells grown in plastic wells, EGTA treatment was monitored by microscopy and continued until cells became dome shaped (21). For cells grown on filters, EGTA treatment was continued until the TER reached the baseline levels measured for filters without cells that were treated in an identical manner. The medium was monitored for excessive cell detachment by counting cells in the supernatant by hemocytometry and comparing the counts to those for untreated cells. Little or no cell detachment was noted during any of these experiments.

(ii) Growth of cells in low-calcium medium. Cells were plated at high density (split ratio, 1:1) in low-calcium medium (LCM) prepared with sMEM (5 μM Ca^{2+}; Gibco BRL Life Technologies, Inc., Grand Island, N.Y.) instead of MEM (which contains 1.8 mM Ca^{2+}) and with dialyzed serum (University of California Cell Culture Facility). LCM supports cell growth and does not affect cell attachment to the substratum but contains inadequate calcium for tight junction formation (17). Cells grown in LCM did not form TER above baseline levels. After 11 h in LCM, the medium was replaced with regular MEM in half of the cell samples for 5 h to restore Ca^{2+} to normal levels and to allow tight junctions to form (18). Cells that were switched to MEM developed TER levels normal for confluent cells; cells that were not switched did not.

Invasion assays. Cells grown on filters or in plastic wells were inoculated with 150 μl or 200 μl, respectively, of bacterial suspension containing 10^6 or 10^7 CFU/ml in MEM that was buffered with 1 M HEPES-NaOH (pH 7.6), 0.35 g of $NaHCO_3$, and 6 g of bovine serum albumin (Sigma)/liter. Cells were incubated at 37°C for 3 h. Bacterial invasion of cells was quantified by gentamicin survival assays; survivors of 2 h of treatment with 0.5 ml of a solution containing 200 μg of gentamicin (Sigma)/ml of culture were enumerated by viable bacterial cell counts after cells were washed in phosphate-buffered saline to remove the antibiotic and after cell lysis by a 15-min treatment with 0.5 ml of 0.25% Triton X-100 (Sigma). In experiments in which cells were grown on filters, the filter was cut away from the filter holder prior to cell lysis in Triton X-100. At least three wells were used for each group, and all experiments were repeated at least three times.

Association assays. The total number of bacteria (the sum of invaded and adherent bacteria) that had associated with cells during the 3 h of incubation with bacteria was determined by methods similar to those used in invasion assays except that cells were not treated with gentamicin prior to cell lysis with Triton X-100. All association assays were performed with cells that had been grown on filters, which were cut from their holders prior to cell lysis so that bacteria that had adhered to the plastic surrounding the filter were excluded from the enumeration process.

Cytotoxicity assays. Cytotoxicity was determined by trypan blue exclusion assays that were carried out by inoculating epithelial cells with bacteria as described above and then adding 0.4% trypan blue (Sigma) in Ham's F-12 to each well to visualize injured cells. Cytotoxicity was assessed by two techniques: (i) counting stained foci and (ii) assessing the percentage of stained cells within each well under the microscope. Results obtained by these methods of scoring cytotoxicity have been found to correlate well with the results of quantitative assessment by chromium release assays (12).

Cell differentiation. Invasion of migrating cells was compared to invasion of confluent monolayers. MDCK cells were plated at various densities in plastic wells so that invasion assays could be simultaneously performed with cells at different stages of confluence. Levels of confluence in each well were assessed by visual inspection prior to the addition of bacteria. The total number of bacteria that had invaded cells in each well was determined by gentamicin survival assays as described above. The number of epithelial cells per well and the number of bacteria that invaded per cell were calculated after collecting cells in half of the wells and counting epithelial cells by standard hemocytometric techniques.

The role of extracellular calcium in *P. aeruginosa* invasion of cells. MDCK cells were grown in plastic wells. In these experiments, bacteria were added in the presence of LCM without serum or antibiotics. Control samples received MEM containing regular calcium levels with the bacterial inoculum. All cells in both the treatment and control groups were pretreated with EGTA prior to the addition of bacteria to disrupt cell polarity and thus eliminate differences in basolateral surface exposure between the two treatment groups. Susceptibility to invasion by strain 6294 was compared for the cells that were exposed to low and regular calcium levels.

Statistics. The *t* test and analysis of variance (ANOVA) were used to analyze the data.

RESULTS

***P. aeruginosa* invasion of various types of epithelial cells following EGTA pretreatment of cells to disrupt polarity.** EGTA pretreatment of cells increased *P. aeruginosa* invasion of all cell types that were tested (Table 1). The TER of MDCK cells grown on filters was approximately 200 ohms/cm^2; TER of filters without cells that were treated in an identical fashion was approximately 110 Ω/cm^2. EGTA treatment decreased the TER of filters with cells to the baseline levels observed for filters without cells at a concentration of 30 mM when cells were treated for 30 min. This was similar to the findings of previous studies (34). EGTA treatment of MDCK cells was found to increase invasion between 6- and 30-fold in all experiments (Table 1).

Initially MDCK cells were grown on filters, since this method of growth was thought to optimize polarity across the cells. However, MDCK cells grown on plastic tissue culture well surfaces responded similarly to EGTA treatment (Table 1), suggesting that cells grown in this fashion were also polarized for susceptibility to *P. aeruginosa* invasion.

The increased levels of bacterial invasion as measured by gentamicin survival assays following EGTA treatment of epithelial cells were not due to trapping of bacteria between cells that might have recovered their tight junctions during the assay. Control experiments were performed in which cells were given a second bout of EGTA treatment to ensure that tight junctions were disrupted just prior to killing extracellular bacteria with gentamicin. Similar numbers of bacteria survived gentamicin killing when cells were retreated just prior to the addition of gentamicin ($3.0 \times 10^4 \pm 0.5 \times 10^4$) as survived when cells were only pretreated with EGTA ($2.4 \times 10^4 \pm 0.7 \times 10^4$).

Similar findings were noted for the other epithelial cell types after EGTA treatment. The results showed that those cells that developed the highest TER levels had the lowest pretreatment susceptibility to invasion and the greatest increase in invasion following EGTA treatment (Table 1). Primary cultures of nasal epithelial cells developed a TER of nearly 2,000 Ω/cm^2, and there was very little bacterial invasion of these cells before EGTA treatment; after treatment invasion levels increased nearly 1,000-fold.

TER across cells was monitored at each stage throughout the bacterial interaction assays. The results presented in Table 1 are from experiments in which EGTA treatment was continued for long enough to reduce TER across cells to baseline levels for the duration of the bacterial interaction assays. When we used these methods, increases in bacterial invasion of all cell types were accompanied by corresponding increases in bacterial adherence to cells (Table 1). However, experiments with human corneal epithelium (HCE) cells in which the length of EGTA treatment was varied showed that shorter EGTA treatment periods increased bacterial invasion of cells without affecting the number of bacteria that adhered to cells. A 45-min treatment period increased invasion ($3.0 \times 10^3 \pm 1.2 \times 10^3$ to $2.3 \times 10^4 \pm 0.8 \times 10^4$; $P = 0.03$) yet did not significantly alter bacterial association with cells ($9.0 \times 10^4 \pm 1.6 \times 10^4$ to $8.4 \times 10^4 \pm 1.1 \times 10^4$; $P = 0.77$). In those experiments, it was found that the TER across cells had returned to levels that were close to pretreatment levels by the end of the bacterial invasion assay (185 ± 3).

The concentration and time of EGTA treatment required to disrupt tight junctions and thus reduce TER to baseline levels was not correlated with initial TER (Table 1). Indeed, respiratory epithelial cells developed the highest TER yet required the lowest EGTA concentration.

***P. aeruginosa* invasion increased when MDCK cells were grown in LCM to inhibit the development of polarity.** Growth of MDCK cells in LCM inhibits the development of tight junctions and thus of cell polarity (17). Cells that have been grown in LCM can form tight junctions only if calcium levels are restored for a sufficient time (18). This can be achieved with MDCK cells by switching LCM-grown cells to medium containing 1.8 mM Ca^{2+} (MEM) for 5 h. Cells grown only in LCM were compared to MEM-switched cells for their susceptibility to invasion by strain 6294. In these experiments, regular calcium levels were restored to all cells at the time when bacteria were added. MDCK cells grown only in LCM (not polarized) were more susceptible to *P. aeruginosa* 6294 invasion than cells switched to MEM for 5 h (polarized) ($1.6 \times 10^4 \pm 0.1 \times 10^4$ and $1.4 \times 10^3 \pm 0.2 \times 10^3$ CFU, respectively; $P = 0.004$). These results complement those found with EGTA-pretreated cells to suggest that the basolateral cell surfaces of MDCK cells are more susceptible to *P. aeruginosa* invasion than the apical surfaces.

***P. aeruginosa* invasion of MDCK epithelial cells was not affected by low calcium.** Although growth of cells in LCM was found to enhance *P. aeruginosa* invasion of MDCK cells, performing the bacterial invasion assay in the presence of LCM did not affect uptake. Neither bacterial association with cells nor bacterial entry into cells was inhibited when calcium levels were reduced to 5 μM while cells were incubated with bacteria. The number of bacteria that associated with cells was $2.0 \times 10^5 \pm 0.2 \times 10^5$ in low calcium and $2.4 \times 10^5 \pm 0.1 \times 10^5$ in regular calcium ($P = 0.2$). The number that invaded cells was $6.9 \times 10^4 \pm 0.4 \times 10^4$ in low calcium compared to $6.5 \times 10^4 \pm 0.7 \times 10^4$ in regular calcium ($P = 0.6$). These experiments were done with cells that had been EGTA-pretreated to disrupt tight junctions in order to level the playing field; i.e., all cells were exposing their basolateral cell surfaces before bacteria were added. In experiments in which one or the other of these pretreatments was not done, bacterial invasion was greater in LCM (data not shown), probably because basolateral cell surfaces were beginning to become exposed during the assay while cells to which bacteria had been added in regular calcium levels (1.8 mM in MEM) would have retained the integrity of their tight junctions all the way through to the end of the assay.

Cell polarity affected susceptibility to cytotoxicity by cytotoxic *P. aeruginosa* strains. Both MDCK cells and HCE cells were tested for their susceptibility to cytotoxicity by the cytotoxic strains 6206 and PA103 after EGTA treatment. Cells grown on filters or in plastic wells were examined with an inoculum of 1.5×10^5 CFU per well.

Both cell types became more susceptible to killing following EGTA treatment irrespective of whether they were grown on filters or on plastic; the effect was to increase the size and the number of foci of dead and dying cells as assessed by trypan blue exclusion assays. In some areas the foci were joined to form confluent areas of dead cells, an effect that was not observed with cells that were not pretreated with EGTA. A typical experiment showing bacterium-induced cytotoxicity of MDCK cells grown on plastic tissue culture wells before and after EGTA treatment is shown in Fig. 1. In control experiments it was shown that cells that were EGTA treated and exposed to a noncytotoxic *P. aeruginosa* strain, such as 6294, or that were not inoculated with bacteria did not stain with trypan blue.

MDCK cells were also found to be more susceptible to killing by strain 6206 if the epithelial cells were grown in LCM. Cells that were switched to MEM for 5 h prior to the addition of bacteria were found to have approximately fivefold fewer

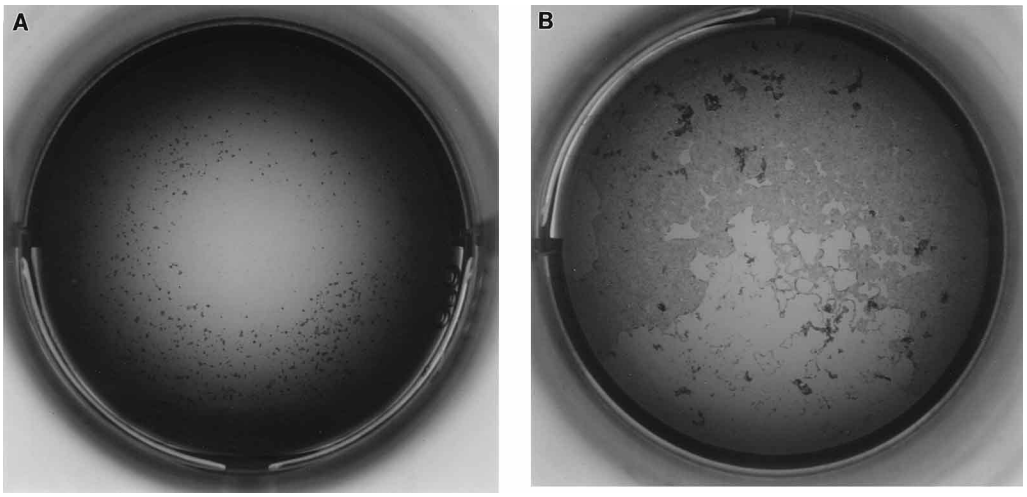

FIG. 1. Effect of EGTA treatment (30 mM for 30 min to disrupt tight junctions and cell polarity) on MDCK cell susceptibility to *P. aeruginosa* cytotoxicity (strain 6206; inoculum, 1.5×10^5). Cells were grown in plastic wells. Cytotoxicity was enhanced by EGTA treatment. (A) Cells were not pretreated with EGTA, and small foci of cytotoxicity were observed. (B) Cells were pretreated with EGTA to disrupt tight junctions; foci of cytotoxicity often joined to form homogeneous areas of dead cells. Foci of cytotoxicity were not observed if bacteria were not added, irrespective of whether cells were EGTA pretreated.

foci of dead cells (18 ± 3) than did cells that were not switched to MEM until the bacteria were added (62 ± 5) ($P < 0.05$).

Effect of cell differentiation on *P. aeruginosa* invasion of MDCK epithelial cells. Differentiation of cells affected the efficiency of *P. aeruginosa* invasion (Fig. 2). Cells were plated at different concentrations to produce cultures with different levels of confluence. Cells that were subconfluent took up 13 times as many bacteria per cell as confluent cells. Cells that were sparsely plated took up 33 times as many bacteria per cell as confluent cells (ANOVA, $P = 0.001$). Similar results were found with HCE cells (data not shown).

Foci of cytotoxicity were not observed with cells that were not confluent. This finding suggested that foci may only form when cells are adjacent to one another.

ConA^r cells are resistant to *P. aeruginosa* virulence factors because they are highly polarized. ConA^r MDCK cells that were selected for their resistance to killing by concanavalin A were previously found to be resistant to killing by the cytotoxic *P. aeruginosa* PA103 (1). ConA^r cells were tested for their susceptibility to bacterial invasion by the noncytotoxic *P. aeruginosa* 6294. The results showed that ConA^r cells were less susceptible to *P. aeruginosa* invasion than regular MDCK cells. The number of bacteria recovered from MDCK cells with an inoculum of 1.5×10^5 CFU per well for 3 h was $8.8 \times 10^3 \pm 0.5 \times 10^3$, while only 430 ± 110 could be recovered from within ConA^r cells ($P = 0.0001$). When the inoculum was increased 10-fold, bacterial invasion of MDCK cells increased proportionally to $4.6 \times 10^4 \pm 0.5 \times 10^4$ but did not increase for ConA^r cells (670 ± 160).

During the course of these experiments the ConA^r cells were found to produce extremely high TER levels of up to 2,000 Ω/cm^2 compared to those of the MDCK cells, which were around 200 Ω/cm^2. Our findings with respect to cell polarity and susceptibility to *P. aeruginosa* led us to test the hypothesis that the ConA^r cells did have the necessary receptors for *P. aeruginosa* interactions but that these cells were more efficient at establishing polarity than MDCK cells. If this were so, then the basolateral membrane constituents that interact with *P. aeruginosa* virulence factors would be more segregated from the apical cell membrane in ConA^r cells than they are in regular MDCK cells. To test this hypothesis, ConA^r cells were EGTA treated to expose their basolateral membranes. EGTA treatment was found to restore susceptibility to invasion by strain 6294 (Fig. 3) and to cytotoxicity by 6206 or PA103 (Fig. 4). The tight junctions of the ConA^r cells were so resilient that EGTA treatment had to be continued for twice as long as for MDCK cells to reduce TER to baseline levels. Furthermore, it was necessary to perform invasion assays in the presence of LCM in order to prevent the TER from rising to pretreatment levels during the invasion assays.

Although polarity effects explain the reduced susceptibility of ConA^r cells to *P. aeruginosa* virulence factors, polarity effects did not explain their resistance to killing by concanavalin A. ConA^r cells remained resistant to concanavalin A even when they were EGTA treated, while even untreated MDCK cells were killed (data not shown). The results suggest that

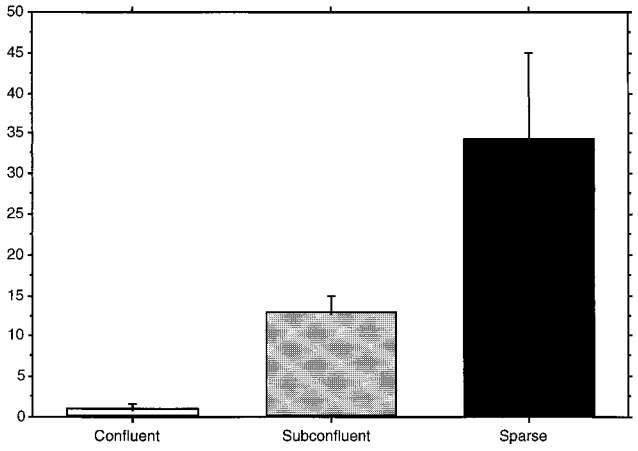

FIG. 2. Epithelial cell differentiation affected the efficiency of *P. aeruginosa* invasion of MDCK cells. Cells were plated at different concentrations to produce cultures with different levels of confluence, and the results showed that sparse cells were more susceptible to invasion than confluent cells (ANOVA, $P = 0.001$). Sparse, 1× (~10^4 cells/well); subconfluent, 3×; confluent, 7.5×. Since there were different numbers of cells in each well, relative invasion levels were standardized such that the amount of invasion for a fixed number of cells was rated as 1 for confluent cells. Error bars indicate standard deviations.

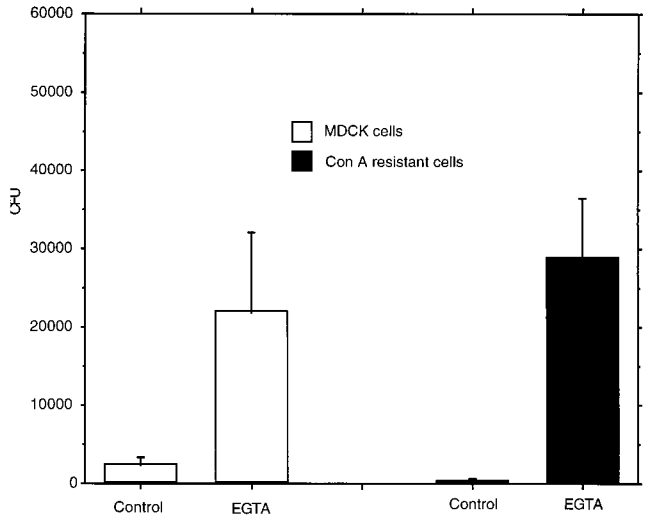

FIG. 3. MDCK cells or ConA^r cells were compared for their susceptibility to invasion by *P. aeruginosa* 6294 with or without EGTA pretreatment of cells. ConA^r cells were less susceptible to bacterial invasion than MDCK cells ($P = 0.01$) but became as susceptible as MDCK cells after EGTA treatment ($P = 0.39$) (MDCK cell invasion increased approximately 10-fold, while that of ConA^r cells increased about 100-fold). In these experiments EGTA treatment reduced the TER of MDCK cells from 202 ± 2 to 96 ± 4 and reduced that of ConA^r cells from 718 ± 70 to 110 ± 5. After EGTA pretreatment, bacteria were added in LCM to prevent the re-formation of tight junctions during the assay. All cells were grown on filters, and the inoculum was 1.5×10^5 CFU per well. Error bars indicate standard deviations.

ConA^r cells, although they are likely to be glycosylation-defective, do have the necessary receptors for susceptibility to *P. aeruginosa* virulence. The ConA^r cells are an example of how the efficient maintenance of cell polarity can protect cells from bacterial virulence mechanisms even though the cells carry susceptibility factors for bacterial-cell interactions on their membranes.

DISCUSSION

In this study, various methods were used to show that exposed basolateral membranes of polarized epithelial cells are more susceptible to *P. aeruginosa* invasion and cytotoxicity than the apical membranes. These results were not explained by increases in exposed membrane surface area. The surface area ratio of basolateral to apical surfaces of MDCK cells grown by the methods used in this study is approximately 1:1 (5, 34), so increases of greater than twofold are not surface area related. For MDCK cells susceptibility to invasion increased between 6- and 30-fold in all experiments. For ConA^r cells invasion increased 100-fold after EGTA treatment.

With shorter exposure of cells to EGTA, susceptibility to bacterial invasion increased without affecting the ability of bacteria to bind to cells, showing that the efficiency of bacterial invasion can be increased without significant increases in bacterial binding to cell membranes. The reason why longer periods of EGTA treatment also increased bacterial adherence was not clear; however, it is possible that longer exposure may have exposed filter surfaces to which bacteria could then adhere.

Migrating epithelial cells are not polarized, since they have no tight junctions. These cells were found to be more susceptible to *P. aeruginosa* invasion than confluent cells. Similar findings were recently noted for *L. monocytogenes* (15). Interestingly, cells that were not confluent appeared to be less susceptible to cytotoxicity; i.e., foci of dead cells were not observed. This suggested that cellular factors involved in susceptibility to cytotoxicity were only present on the cell membrane surface when cells were in close proximity to one another. The expression of these factors is not likely to be calcium dependent and is independent of tight junction formation, since cells grown juxtaposed in low calcium were found to be hypersusceptible to cytotoxicity. These results suggest that cell-to-cell proximity may be important for focus formation.

In a recent paper it was noted that MDCK cells that were selected for resistance to the lectin concanavalin A (ConA^r cells) were also resistant to *P. aeruginosa* cytotoxicity (1). Since certain carbohydrates are thought to be receptors for *P. aeruginosa* (31), this result suggested that the resistance of these cells to killing by the bacteria was due to glycosylation defects. In this study, data is presented to show that ConA^r cells are also resistant to *P. aeruginosa* invasion.

EGTA treatment of ConA^r cells was found to restore their susceptibility to both cytotoxicity and invasion, which then becomes indistinguishable from the susceptibility of EGTA-treated MDCK cells. This result suggests that the ConA^r cells,

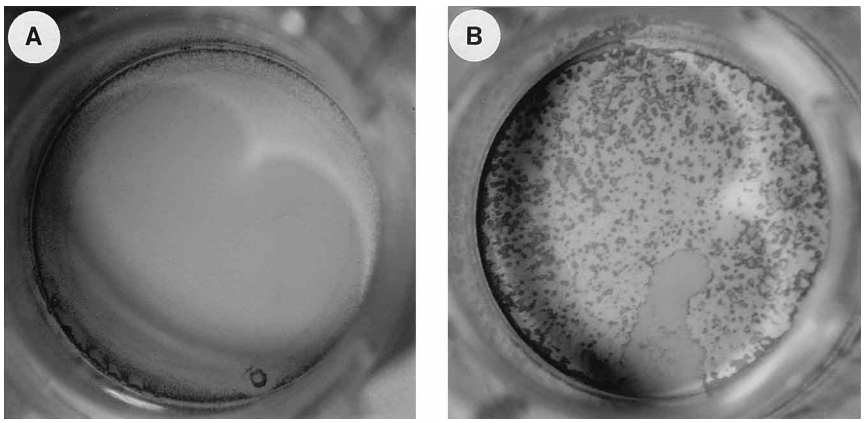

FIG. 4. ConA^r cells became susceptible to cytotoxicity by strain 6206 after EGTA pretreatment. Cells were grown on filters and exposed to 1.5×10^5 CFU for 3 h and then stained with trypan blue to detect dead or dying cells. Foci of cytotoxicity were not observed if bacteria were not added, irrespective of whether the cells were EGTA pretreated. (A) Cytotoxicity was not observed when bacteria were added without EGTA pretreatment of cells. (B) EGTA pretreatment restored cell susceptibility to cytotoxicity. See text for details of methods.

although glycosylation defective, were not missing a receptor(s) for *P. aeruginosa* cytotoxicity and invasion. Instead, it indicates that the presence, or the accessibility, of receptors was more efficiently polarized to the basolateral cell membrane in the ConA^r cells than in the MDCK cells. Polarity effects did not explain their resistance to concanavalin A: ConA^r cells remained resistant to killing by concanavalin A even when they were EGTA treated.

Cell polarity is determined both by the integrity of tight junctions and by the polarized delivery, retention, and removal of cell membrane molecules. Protein delivery and retention is never completely polarized. For example, the polymeric immunoglobulin receptor is primarily delivered from the golgi to the basolateral surface, but about 10% of molecules are mistargeted to the apical surface (24). Furthermore, the efficiency of polarized delivery of a particular membrane component can vary among different cell types. This would explain why polarized confluent epithelial cells (e.g., MDCK cells) were somewhat susceptible to *P. aeruginosa* virulence mechanisms and why there were differences in susceptibility between different epithelial cell types tested in this study.

The increased polarity of ConA^r cells for susceptibility to *P. aeruginosa* virulence factors could have been due to an increase in the effectiveness of their tight junctions and/or in the polarity of delivery and retention of membrane factors involved in susceptibility to *P. aeruginosa* infection. ConA^r cells had to be treated with EGTA for twice as long as MDCK cells to reduce the TER to baseline levels, and the 3-h bacterial invasion assays had to be done in LCM to prevent the tight junctions from reforming. These difficulties suggested that the cells may have had very resilient tight junctions.

Although all of the epithelial cells studied became more susceptible to both cytotoxicity and invasion by *P. aeruginosa* strains once they lost their polarity, these findings do not indicate any particular receptor(s) for these events, nor do they suggest that the two phenomena occur via the same receptor(s). Increased susceptibility to *P. aeruginosa* in less well polarized cells may indicate that receptors for bacterial interaction are more abundant on the basolateral surfaces. Alternatively, the results could be explained by decreased accessibility to receptors on the apical surface; i.e., there may be factors on the apical membrane surface that inhibit bacterial interaction with receptors. We have found that in the cornea, ocular mucin is able to inhibit bacterial adherence to corneal epithelial cells (10). The cell surface glycocalyx is composed of mucin and is only found on the apical membrane of surface corneal cells (16).

P. aeruginosa-induced epithelial cell cytotoxicity begins as a few expanding foci of dying cells that eventually coalesce to affect the whole cell population (1, 12). Apodaca et al. studied *P. aeruginosa* cytotoxicity by confocal microscopy with strain PA103 and noted bacteria underneath dead MDCK cells in these foci but not under cells that were unaffected. Using time lapse videomicroscopy of focus formation by the cytotoxic strains PA103 and 6206, we have observed bacteria killing a single central cell and then the spreading of bacteria outward underneath adjacent cells in the foci; death of a particular cell coincided with the presence of bacteria underneath that cell (unpublished results). These observations suggest that once the central cell is killed by bacteria, they then have access to the basolateral surface of adjacent cells and therefore the killing can spread. It can be inferred from these observations that an occasional cell is attacked by bacteria while the cell layer is still intact, suggesting that although most cells in a population are polarized for their susceptibility to *P. aeruginosa* cytotoxicity, an occasional cell is less well polarized. The foci appear to begin with the killing of these cells. Cells in the ConA^r cell population all appeared to be efficiently polarized for *P. aeruginosa* receptors, since cytotoxicity was not observed unless cells were EGTA treated. The behavior of these cells demonstrates how efficient cell polarity can protect epithelial cells from bacterial attack.

The authors of a recent publication suggested that the cystic fibrosis transmembrane regulator (CFTR), which is the protein that is defective in patients with cystic fibrosis, is the receptor for *P. aeruginosa* invasion (26). CFTR is apically expressed on epithelial cells, yet our findings suggest that the basolateral cell surface is more susceptible to *P. aeruginosa* invasion. These two findings appear to be contradictory, unless exposure to bacteria alters the distribution of CFTR on cell membranes.

We recently published results showing that there is more *P. aeruginosa* invasion into corneal epithelial cells that are in the process of exfoliation than into epithelial cells that are attached to the cornea (11). This result suggested a role for exfoliation of surface corneal epithelial cells, and their rapid removal from the eye, in defense against infection by *P. aeruginosa*. Subsequently, Pier et al. proposed a similar mechanism for defense against *P. aeruginosa* infection in the lung (26). Selective adherence of *P. aeruginosa* to desquamating epithelial cells during tracheal infection has been observed by electron microscopy (4). The preference for *P. aeruginosa* invasion of basolateral membranes suggests a mechanism for selective bacterial uptake by cells that are in the process of exfoliation, since exfoliating cells lose their tight junctions before they become detached.

The consequences of bacterial entry into basolateral membranes of cells that are not in the process of exfoliation may be entirely different. Bacteria inside cells are relatively protected from the immune system, while they may remain capable of stimulating the release of inflammatory mediators (7). Indeed, various factors that expose basolateral membranes of cells other than those in the process of exfoliation happen to be risk factors for infection; these include injury, inflammation, hypoxia, and edema (31).

Other investigators have found a similar preference for basolateral entry into host cells by certain other bacteria and even some viruses. These include *S. flexneri* (25), *Klebsiella pneumoniae* (19), *L. monocytogenes* (15), poliovirus (35), vesicular stomatitis and Semliki Forest virus (14), canine parvovirus (3), the DNA vaccinia virus (29), and most recently herpes simplex virus (34). The growing list of microorganisms that share this behavior suggests that epithelial cell polarity may play a general role in defense against invasion of cells by microorganisms. Our findings suggest that cell polarity not only protects cells from bacterial invasion, it also contributes to defense against bacterium-induced cytotoxicity.

Many of the receptors on eukaryotic cell surfaces that microbes interact with are essential for host cell function. It would not be surprising if the evolutionary process prevented the survival of epithelial cell surfaces with exposed receptors for infection on their apical cell surfaces. Indeed, the inability to infect or kill cells via their apical cell membranes may contribute to the distinction between opportunistic species like *P. aeruginosa* and outright pathogens.

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