PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 173, 588–597 (1983)

Antibiotic and Nonantibiotic Ionophores Can Alter Bacterial Adherence to Mammalian Cells[1] (41692)

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Abstract. Epithelioid (HeLa) and fibroblastic (L) cells in culture incubated for 18 hr with the ionophores amphotericin B and amiloride were noted to bind significantly more and less bacteria, respectively, than control cells incubated without ionophores. These effects were related to dose and incubation length and were present at concentrations approximating those *in vivo* after administration of maximal doses of these drugs given to humans therapeutically. Electron microscopy of both receptor cell lines revealed increased length and number of cellular projections in the amphotericin-treated cells and flattening and loss of membrane individuality in the amiloride-treated cells. These findings could explain the differences in subsequent bacterial binding. The ionophores nifedipine and verapamil which block calcium transport in cells which have calcium channels did not alter bacterial binding to these receptor cells or bacterial binding to calcium channel-containing myoblasts (in culture). These data suggest that certain ionophores could alter bacterial colonization and infection in the host indirectly by altering bacterial binding; however, the clinical significance of these findings remains to be determined.

The adherence of bacteria to mammalian surfaces may precede colonization, infection, or both involving the heart, gastrointestinal, respiratory, and genitourinary tracts (1–3). Substances which decrease adherence (e.g., antibodies, antibiotics) may lessen this process (3) and others (e.g., zinc, iron) may accentuate the phenomenon (4, 5). *In vivo* experiments with antibiotics, vaccines to induce antibody formation, introduction of compounds which preferentially bind bacteria, and other treatments have suggested that active control of bacterial adherence could be useful in prevention, treatment, or both of certain infections (3, 6–9). Conversely, exposure to agents that increase bacterial adherence may be a potential factor in increased susceptibility to infection (3–5).

One of the parameters that may play a crucial role in the adherence of microorganisms to surfaces is the surface charge of the involved cells (10–12). Some ionophores are administered systemically to humans for a variety of purposes. Amphotericin B is an antifungal antibiotic that alters the permeability of sensitive cells (including epithelial cells) to various ions such as potassium (13–15). Amiloride inhibits chloride secretion and sodium absorption by epithelial cells and is used as a diuretic (and has been used topically to test respiratory epithelial function of patients with cystic fibrosis) (16, 17). Nifedipine and verapamil decrease calcium influx into excitable cells which have calcium channels and are used as cardioactive drugs (18). These ionophores could effect bacterial adherence, colonization, and infection directly by altering the surface charge of receptor cells (if altered ion flux is associated with changes in ion accumulation at the cell surface) or indirectly via other cellular changes. We decided to systematically study the effect of ionophores in current clinical usage on epithelioid, fibroblastic, and myoblastic (with calcium channels) cells' ability to bind bacteria.

Methods. *Bacteria.* Freshly isolated clinical specimens were maintained on trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) plates. Enterobacteriaceae were identified by the A.P.I. system (Analytab Products, Plainview, N.Y.) and staphylococci by gram stain, colonial morphology on blood agar plates, and coagulase activity. The Enterobacteriaceae were heavily piliated with predominantly morphological type I pili (5)

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[1] Presented at the American Federation for Clinical Research, Southern Section meeting in New Orleans, La., January 1983.

0037-9727/83 $1.50

that cause D-mannose-sensitive guinea pig erythrocyte hemagglutination. Bacteria were radiolabeled by incubation at 37°C for 18 hr in Eagles' minimal essential medium (MEM) with Earle's salts (Gibco, Grand Island, N.Y.) for the Enterobacteriaceae or Mueller–Hinton Broth (Difco, Detroit, Mich.) for the staphylococci. Methyl[^{3}H]thymidine (New England Nuclear, Boston, Mass.) with specific activity of 80.3 Ci/mmole was added at 5 μCi/ml. Organisms were collected and washed three times by centrifugation. Bacteria were finally suspended after vortexing to break clusters in 25×10^{-3} *M* *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) (Gibco), pH 7.2. Their concentrations were adjusted spectrophotometrically to 3×10^8/ml; concentrations were verified by quantitative cultures.

Ionophores. Chemically pure amphotericin B (E. R. Squibb and Sons, Princeton, N.J.), verapamil HCl (Knoll Pharmaceutical, Whippany, N.J.), nifedipine (Pfizer Inc., New York, N.Y.), and amiloride hydrochloride dihydrate (Merck and Co., West Point, Pa.) were graciously supplied by the above companies. The ionophores were dissolved in water, except for amphotericin B which was mixed with 0.80 mg sodium desoxycholate/mg amphotericin prior to the addition of water and nifedipine which was first dissolved in ethanol (final volume ethanol less than 0.05%). Controls included water and sodium desoxycholate. These compounds were incubated for 18 hr (unless otherwise stated) with receptor cells in culture. The ionophores were not exposed to light, which could cause degradation. (Concentrations in the table are about 10 times maximal therapeutic human serum or tissue concentrations.) The buffering capacity of the cell culture media was adequate to keep the pH at 7.2 for all compounds studied.

Mammalian receptor cells. HeLa cells were grown on cell culture petri dishes with 8 cm^2 surface area (Corning, Corning, N.Y.) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. These epithelioid cells from a human carcinoma of the cervix were grown to confluency in pH 7.2 McCoy's modified 5A medium (Gibco) with 5% (v/v) supplemental fetal bovine serum (K. C. Biological, Lenexa, Kans.), 2 m*M* glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. L cells (mouse fibroblasts) were propagated in Eagle's MEM

TABLE I. EFFECT OF IONOPHORES ON SUBSEQUENT BACTERIAL ADHERENCE[a,b]

	Control[c]		Amphotericin B (10.7×10^{-6} *M*)		Amiloride (0.2×10^{-3} *M*)		Verapamil (2.0×10^{-6} *M*)		Nifedipine (0.14×10^{-6} *M*)	
Bacteria	HeLa	L	HeLa	L	HeLa	L	HeLa	L	HeLa	L
Escherichia coli										
−1	1.2 ± 0.1	1.4 ± 0.2	2.3 ± 0.3[d]	2.1 ± 0.2[d]	0.8 ± 0.1[d]	1.2 ± 0.2	1.4 ± 0.1	1.7 ± 0.3	1.1 ± 0.1	—
−2	2.0 ± 0.2	1.1 ± 0.1	2.7 ± 0.3[d]	1.5 ± 0.2[d]	1.2 ± 0.2[d]	0.7 ± 0.2[d]	1.8 ± 0.3	1.2 ± 0.1	—	1.0 ± 0.1
Klebsiella pneumoniae										
−1	2.4 ± 0.2	1.2 ± 0.2	5.3 ± 0.6[d]	1.8 ± 0.2[d]	1.9 ± 0.1[d]	1.0 ± 0.2	2.2 ± 0.3	1.2 ± 0.2	2.6 ± 0.3	—
−2	1.5 ± 0.2	2.4 ± 0.2	2.0 ± 0.2[d]	2.3 ± 0.2	0.9 ± 0.2[d]	1.7 ± 0.1[d]	1.7 ± 0.2	2.2 ± 0.2	—	2.4 ± 0.2
Staphylococcus aureus										
−1	6.9 ± 0.5	8.7 ± 1.0	5.6 ± 0.9	8.4 ± 1.0	4.0 ± 0.3[d]	5.9 ± 0.7[d]	7.6 ± 0.8	8.3 ± 0.9	6.4 ± 0.6	—
−2	7.4 ± 0.4	6.3 ± 0.5	7.1 ± 0.5	6.0 ± 0.6	4.3 ± 0.5[d]	5.1 ± 0.5[d]	7.1 ± 0.7	6.1 ± 0.6	—	6.1 ± 0.6

[a] Ionophores were incubated with receptor cells for 18 hr prior to assay of bacterial adherence to the receptor cells.

[b] Data expressed as mean number adherent bacteria per receptor cell ± SD.

[c] Water. (Desoxycholate results were not statistically different ($P \geqslant 0.3$).)

[d] $P < 0.05$ compared with control.

with Earle's salts and 5% (v/v) fetal bovine serum, 2 m*M* glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Myoblasts (Heart myoblast, rat, ATCC CRL 1446, American Type Culture Collection, Rockville, Md.) were propagated in Eagle's MEM with Earle's salts and 10% (v/v) fetal bovine serum, 2 m*M* glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The total cell protein was calculated after removal of the medium and washing the cells by using a modification of the technique of Lowry *et al.* (19). Within 1 day of confluency of the mammalian receptor cells the ionophores were added. Cell culture plates were then again incubated for 18 hr. Cell viability was determined by trypan blue staining (20) and morphology using phase-contrast light microscopy. When confluent there were $0.82 \pm 0.07 \times 10^6$ HeLa, $1.41 \pm 0.11 \times 10^6$ L, and $8.9 \pm 0.6 \times 10^4$ myoblasts/plate.

Adherence assay. The medium was gently decanted from plates containing confluent and adherent, viable cultured mammalian receptor cells and was replaced with 2 ml of fresh medium without serum and antibiotics. One milliliter Hepes with 3×10^8 bacteria/ml was added and the plates were gently agitated for 45 min at room temperature. Next, nonadherent bacteria were removed from the plates which contained attached receptor cells and adherent bacteria by four rapid rinsings with fresh medium (final washing contained counts per minute (CPM) not significantly above background). Remaining plate contents were solubilized with 0.1 *N* NaOH and counted in a liquid scintillation counter after neutralization to pH 7 and the addition of xylene–surfactant-based scintillation fluid; calculated counting efficiency was 41%. The CPM of known quantities of each bacterial isolate (averaging about 10^3 cfu/cpm) were used to convert CPM to number of bacteria. Under these incubation conditions, the tissue-cultured cells remained viable and confluent, and they attached to the plate surface (as verified by phase-contrast light microscopy and trypan blue staining) until solubilized with NaOH. This assay is sensitive, specific, and correlates well with light microscopy determination of bacterial adherence, and there is minimal, if any, internalization of bacteria by these receptor cells (3). Adherence is maximal with these incubation conditions and leakage of ^3H from the bacteria and binding of free ^3H to receptor cells is minimal (3, 21).

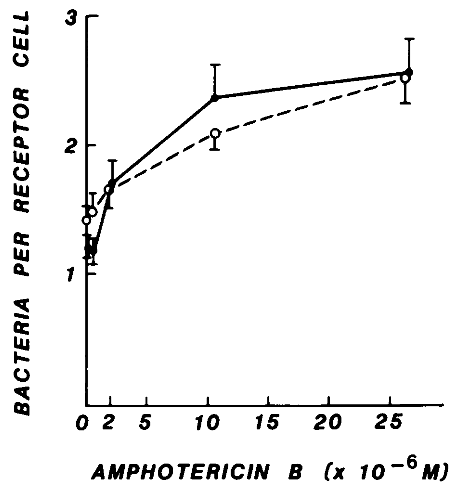

FIG. 1. Effect of amphotericin B concentration on binding of bacteria to receptor cells. HeLa (——) or L (- - -) cells were incubated for 18 hr with amphotericin B in the concentrations noted on the abscissa prior to assay of binding of *E. coli* – 1. $t = 1$ SD.

Electron microscopy. HeLa and L cells were grown to confluency on cover slips in 8-cm² cell culture dishes. Ionophores or controls were added to plates and incubated for 18 hr. The medium was decanted, and the plates were washed with phosphate-buffered saline (pH 7.2, 50 m*M*) warmed to 37°C to prevent shrinkage. Plates were fixed with 1 ml of 3% (v/v) glutaraldehyde (37°C). Samples were rinsed with distilled water, and dehydrated sequentially in ethanol (50% for 5 min, 70% for 5 min, 95% for 5 min, and 100% for 15 to 30 min). Coverslips were then dried in a critical-point drying chamber. Samples were examined with a scanning electron microscope (20 kV) after application of a gold–palladium layer under vacuum.

Statistical methods. Means of assays performed in duplicate were counted as single datum points and results were expressed as the means ± the standard deviation of the means with $N \geq 3$. Statistical significance was determined by analysis of variance and *t* test analysis.

Results. *Amphotericin B.* Exposure of either HeLa or L cells for 18 hr at 10.7×10^{-6} *M* was followed by significantly increased at-

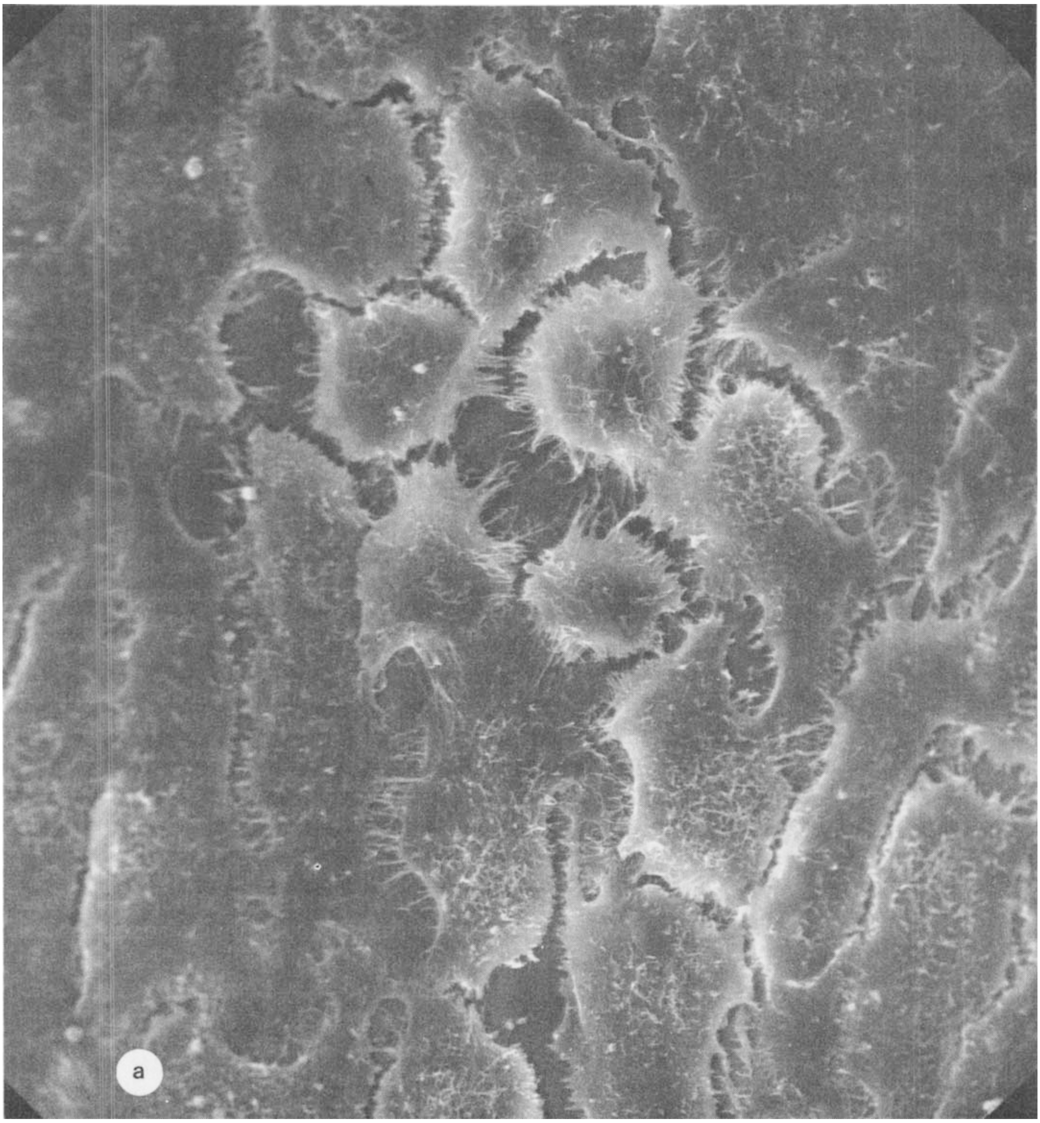

FIG. 2. Effect of ionophores on morphology of HeLa cells. Cells were exposed to water (a), amphotericin B, 10.7×10^{-6} *M* plus desoxycholate (b), desoxycholate (c), or amiloride, 0.2×10^{-3} *M* (d) for 18 hr prior to examination. Magnification, ×1700.

tachment of certain Enterobacteriaceae, but not *Staphylococcus aureus* (Table I). These differences averaged about 50–100%, and represented changes of approximately 10^6 adherent bacteria per plate of receptor cells. The effect was dose-dependent and significant ($P < 0.05$) with at least 2.15×10^{-6} *M* (2 μg/ml) amphotericin B and HeLa cells (Fig. 1). This effect was not present after 1 hr exposure of either bacteria or receptor cells to $2.1–10.7 \times 10^{-6}$ *M* amphotericin B (data not shown). This concentration for 18 hr did not alter receptor cell number, total protein, viability, or morphology under phase-contrast light microscopy (200×). However, both HeLa and L cells were noted on repeated scanning electron microscopy examinations of hundreds of cells to consistently have increased length and number of cellular projections after incubation with $2.1–10.7 \times 10^{-6}$ *M* amphotericin B (Fig.

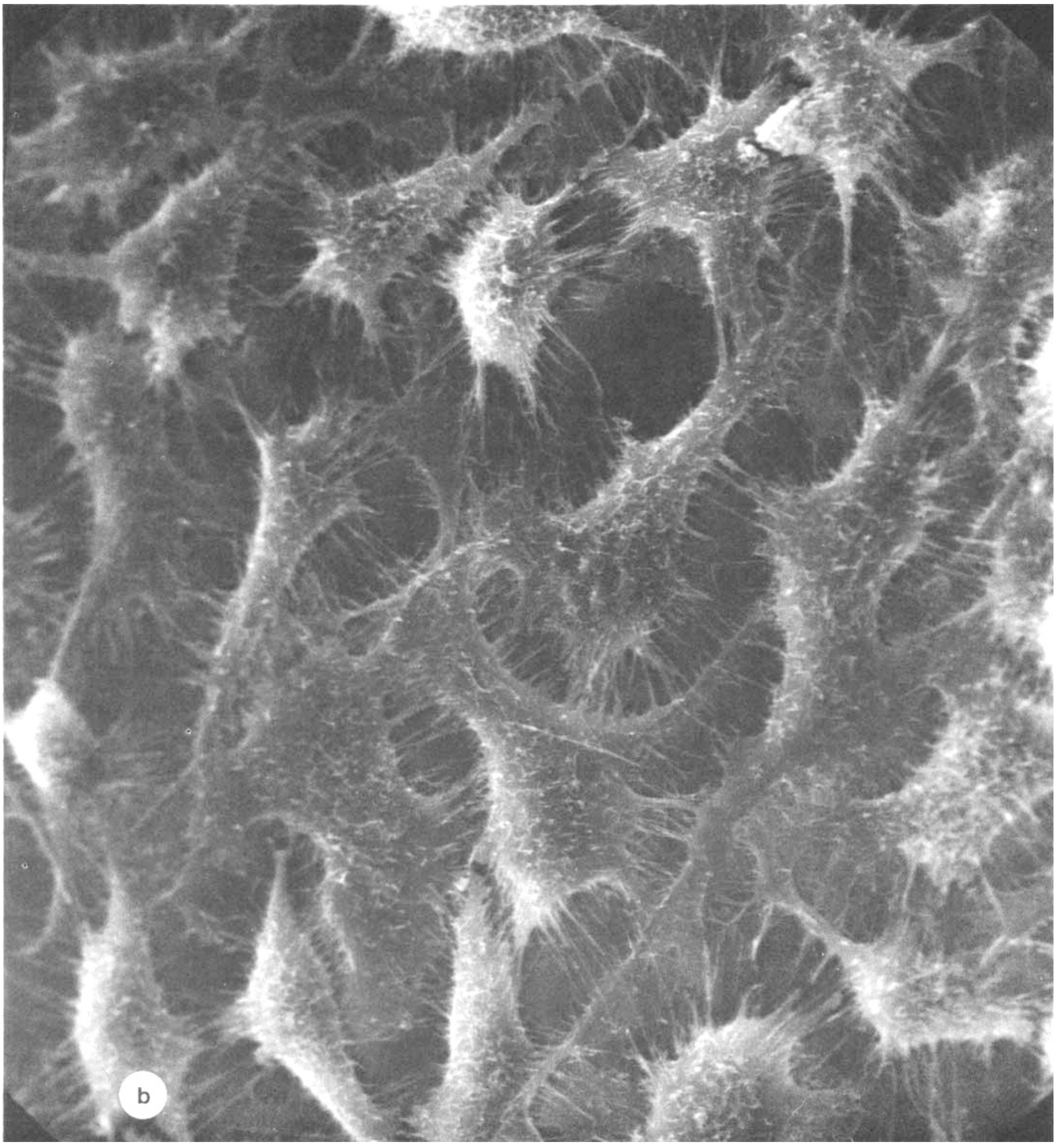

FIG. 2—*Continued.*

2). These effects were more pronounced at the higher concentrations. Neither this change nor the increase in adherence was noted with desoxycholate controls in the concentrations present in up to 10.7 × 10^{-6} *M* commercial amphotericin B for injection (Fig. 2 and Table I).

Amiloride. Amiloride (0.2 m*M*) significantly decreased the attachment of Enterobacteriaceae and *S. aureus* to HeLa and L cells after 18 hr incubation with the receptor cells in 10 of 12 bacteria–receptor cell combinations tested (Table I). There was a definite dose–response effect noted with activity present at concentrations of at least 0.04 m*M* (Fig. 3). One-hour exposure of either bacteria or receptor cells to 0.2 m*M* amiloride did not reproduce this effect (data not shown). Longer incubations with higher concentrations were often toxic to both tissue-cultured cells and bacteria. Eighteen-hour incubations with 0.2 m*M* amiloride resulted in a mean 28%

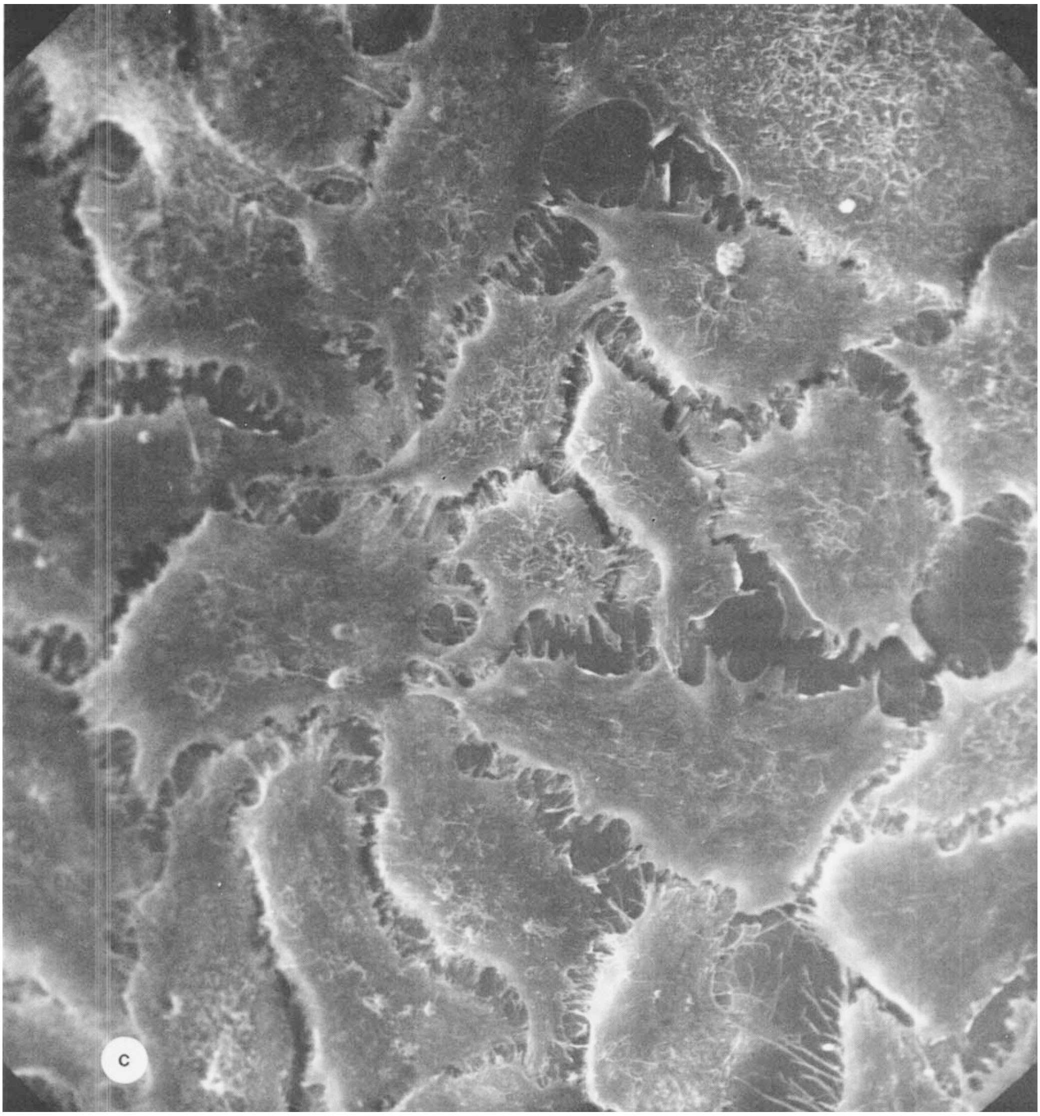

FIG. 2—*Continued.*

($P < 0.05$) and 11% ($P < 0.05$) decrease in HeLa and L cell plate total protein, respectively, compared with control plates; at 0.08 m*M* no decrease was noted ($P \geqslant 0.3$). Viability and morphology under phase-contrast light microscopy (200×) were not affected at any of these concentrations and resembled control plates. Examination of hundreds of random cells on five separate occasions with a scanning electron microscope revealed morphological changes after 18 hr exposure to amiloride (Fig. 2). Both HeLa and L cells showed flattening and a loss of individual cell surface and increased confluency at 0.2 m*M* amiloride, and this was present but less pronounced with 0.04 m*M* amiloride (Fig. 2).

Verapamil and nifedipine. These ionophores did not significantly alter the subsequent adherence of any bacteria tested to HeLa or L cells (Table I and part of data not shown). Verapamil and nifedipine did not alter HeLa or L cell number, viability, total protein, or morphology (phase-contrast light microscopy) after 18 hr incubations with 2×10^{-6} *M* and

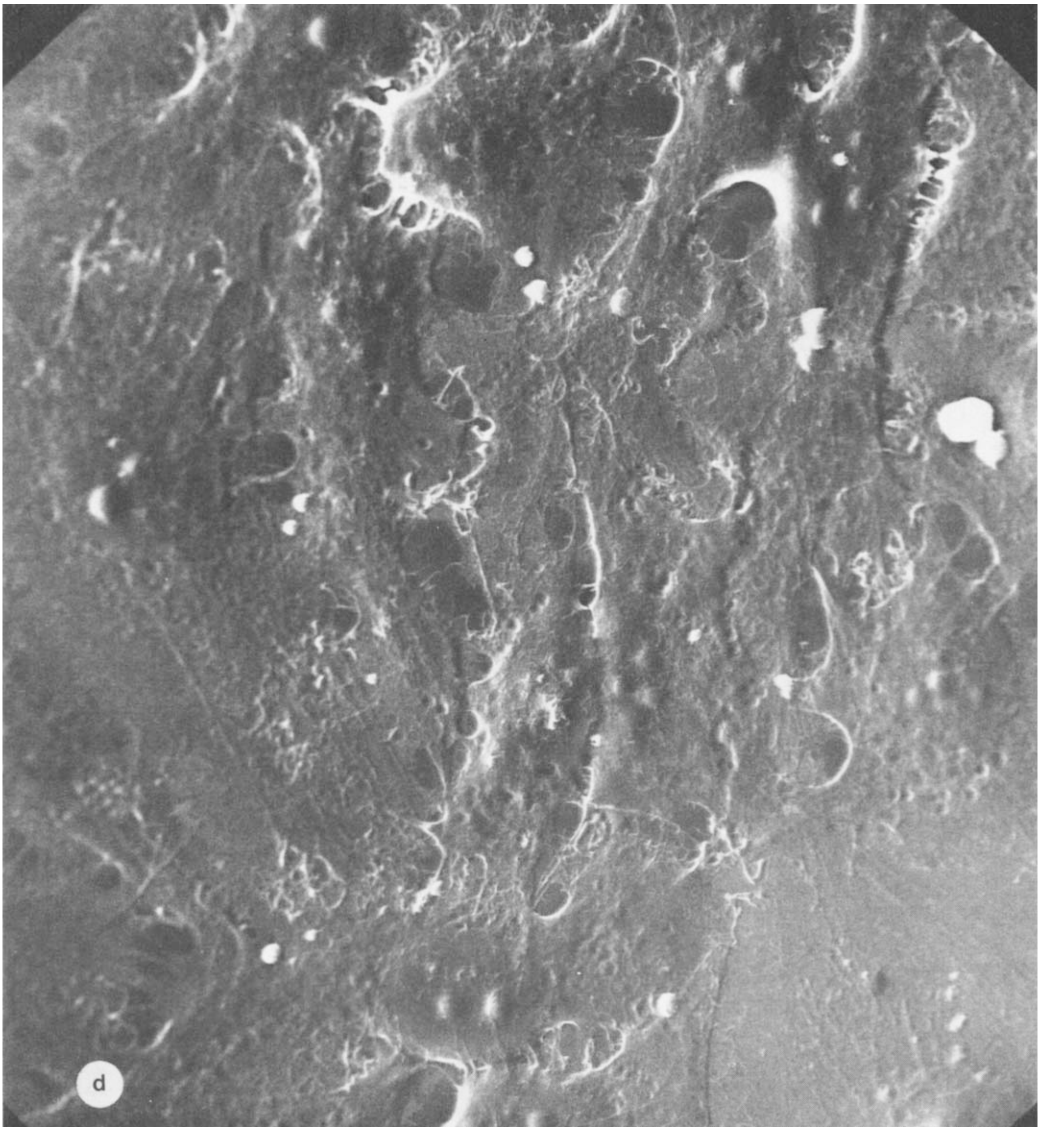

FIG. 2—*Continued.*

0.14×10^{-6} *M* (respectively) concentrations. Random examination of hundreds of receptor cells on multiple occasions with a scanning electron microscope revealed no morphological changes compared to controls after 18 hr exposure to verapamil or nifedipine at the above concentrations. Adherence experiments repeated after incubation of myoblasts with these agents revealed that after 3 or 18 hr incubation with either agent subsequent adherence was not altered consistently in any of the bacteria assayed (Table II).

Discussion. These data show that amphotericin B and amiloride can alter subsequent binding of bacteria to receptor cells after 18 hr incubation. Although ionophores can alter the growth of microorganisms, any such potential activity did not alter subsequent adherence of the bacteria studied to receptor cells after 1 hr incubation (bacteria were never exposed to ionophores for longer than this in any of these experiments) of the bacteria with either amphotericin B or amiloride. Almost all penicillin and streptomycin were removed

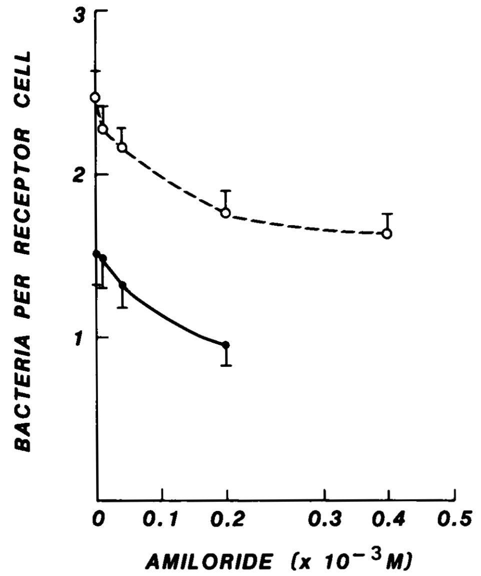

FIG. 3. Effect of amiloride concentration on binding of *K. pneumoniae* −2 to receptor cells. HeLa (——) or L (- - -) cells were incubated for 18 hr with amiloride in the concentrations noted on the abscissa, followed by assay of bacterial binding. *t* = 1 SD.

by washing prior to bacterial exposure and most of the isolates used were highly resistant to these antibiotics. These imply that the changes observed were caused by specific actions of the ionophores on the receptor cells.

Ionophores produce many effects in different cells (14–18). The same morphological changes were noted in two different cell lines in these experiments. Amphotericin B produced increased length and number of cell surface projections and amiloride produced flatter cells with more confluent cell growth and loss of cell individuality. Although amphotericin B is far more active in interactions with fungi, it can have some activity on mammalian cells, probably related to its binding of cell membrane cholesterol (or other membrane sterols) (13–15, 22–24). Consistent with the results reported here, human peripheral blood leukocytes have been noted to show increased adherence to nylon fibers after 60 min incubation with 21.5-53.7 × 10^{-6} *M* amphotericin B (25); however, the mechanism for this was not elucidated. Increased nucleic acid and protein synthesis (and mitogenesis) have been reported to occur in some other mammalian cells following exposure to amphotericin B (26). Similar effects could conceivably result in synthesis of the cellular projections observed in HeLa and L cells. Such effects of amphotericin B may also influence work performed with other cell culture systems using amphotericin B to prevent fungal contamination.

These results suggest that certain Enterobactericeae, but not *S. aureus* attach to receptor cells in greater numbers after exposure of the receptor cells to amphotericin B. This implies specificity of attachment ligands. Enterobacteriaceae often attach to (glycoproteins or glycolipids on) receptor cells with type I (or other) pili (1); lipoteichoic acid may be the adherence ligand for *S. aureus*, though

TABLE II. EFFECT OF IONOPHORES ON SUBSEQUENT ATTACHMENT OF BACTERIA TO MYOBLASTS[a]

	Control		Nifedipine (0.14 × 10^{-6} *M*)		Verapamil (2.0 × 10^{-6} *M*)	
Bacteria	3 hr[b]	18 hr[b]	3 hr	18 hr	3 hr	18 hr
E. coli						
−1	21.2 ± 3.4	20.1 ± 3.3	21.9 ± 3.5	23.2 ± 3.8	23.0 ± 3.1	20.9 ± 3.2
−2	39.7 ± 7.8	42.1 ± 7.9	35.2 ± 7.1	34.7 ± 7.5	36.8 ± 8.1	38.3 ± 7.2
−3	11.9 ± 1.8	12.4 ± 2.0	11.1 ± 1.9	13.1 ± 2.2	12.8 ± 2.1	8.3 ± 1.9[c]
−4	19.1 ± 2.3	18.7 ± 2.2	14.3 ± 2.4[c]	16.8 ± 2.4	19.3 ± 2.2	19.5 ± 2.2
K. pneumoniae						
−3	19.3 ± 3.0	17.1 ± 1.8	18.4 ± 2.8	16.5 ± 2.0	20.1 ± 2.9	17.8 ± 1.7
S. aureus						
−1	48.5 ± 9.8	41.3 ± 11.7	41.7 ± 9.2	35.6 ± 11.8	46.2 ± 10.0	38.8 ± 11.3

[a] Ionophores or controls were incubated with myoblasts prior to assay of bacterial adherence. Data expressed as mean number adherent bacteria per myoblast ± SD.

[b] Length of incubation of ionophores or controls with myoblasts prior to assay of adherence.

[c] $P < 0.05$ compared with appropriate control.

this is controversial (1, 27–29). Although the exact mechanism remains unknown, the morphological changes observed in receptor cell surfaces and surface area could explain these alterations in attachment of bacteria to receptor cells exposed to amphotericin B and amiloride. Thus, the increased cellular projections of the amphotericin B-treated cells could bind more Enterobacteriaceae, and the loss of surface area due to treatment with amiloride may lessen exposed surface receptor sites. Whether leakage of ions or some other mechanism of action of the ionophores was responsible for the morphological changes is not known.

Most known mechanisms of action of ionophores involve alterations in cellular ion permeability (14, 16–18). Bacterial adherence to cells exposed to ionophores could be altered by ionic permeability changes that alter surface charge, morphological changes, a combination of these or other mechanisms. Changes were noted *in vitro* in these experiments that were significant with $2.15 \times 10^{-6}\ M$ amphotericin B and 0.04 mM amiloride or higher concentrations. These levels approximate maximal mammalian serum or tissue levels obtained after administration of these drugs (16, 30–32). Thus, certain ionophores could potentiate or mitigate bacterial adherence to mammalian receptor cells and increase or decrease susceptibility to bacterial infection. The presence and clinical significance of these changes *in vivo,* especially in rapidly dividing cells such as the epithelial cells which line mucosal tracts, remains to be determined.

We thank James Barrish and Linda Babura for expert technical and secretarial assistance. This work was supported by the Veterans Administration.

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Received February 9, 1983. P.S.E.B.M. 1983, Vol. 173.