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Interactions of *Porphyromonas gingivalis* with Epithelial Cells

M. J. DUNCAN,* S. NAKAO,† Z. SKOBE, AND H. XIE‡

Forsyth Dental Center, 140 Fenway, Boston, Massachusetts 02115

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The invasion of gingival epithelial cells by certain pathogenic periodontal bacteria may account for their presence within diseased gingival tissue. To dissect the initial steps of a potential invasion pathway for the periodontal pathogen *Porphyromonas gingivalis*, laboratory and clinical bacterial isolates were tested for their interactions with a human oral epithelial cell line (KB). Several *P. gingivalis* strains immobilized on filters could bind oral epithelial cells. Quantitative adherence assays supported these results. The invasion of epithelial cells by *P. gingivalis* 33277 was measured by assay and confirmed by transmission electron microscopy. These preliminary results demonstrate that certain *P. gingivalis* strains are capable of internalization by human oral epithelial cells in vitro.

Bacterial colonization of gingival tissue and its penetration and destruction are critical processes in the pathogenesis of periodontal disease. Suspected periodontal pathogens possess a wide variety of virulence factors. Adhesins enable bacteria to bind to other bacteria, epithelial cells, and extracellular matrix proteins. Bacteria have also been observed within diseased gingival tissue; so a class of virulence factors which may effect host cell invasion has long been considered a possibility. By entering gingival epithelial cells, bacteria may not only evade the immunological defenses of the host but also reach underlying tissue. It has been proposed that specific gram-negative bacteria play an important role in the etiology of periodontal disease (14). Attention has focused increasingly on a small group of bacteria, which includes *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*, and on the numerous virulence factors they possess (15). Gingival tissue from juvenile periodontitis patients has been quantified with regard to the number of foci which reacted positively with fluorescein isothiocyanate-conjugated *A. actinomycetemcomitans* antisera (2). Samples from a significant number of patients (23%) had stained *A. actinomycetemcomitans* antigen located within epithelial cells. Subsequently, it was reported that strains of *A. actinomycetemcomitans* could invade oral epithelial cells in vitro (16), and the conditions for, and the efficiency of, invasion were characterized (9). Several reports describe the binding of the gram-negative anaerobe *P. gingivalis* to epithelial cells (1, 6, 10), and this organism has also been seen within gingival tissue (11). The latter observation suggests that *P. gingivalis* may also have a means of passing through the epithelial cell barrier. We wish to understand, at the molecular level, the mechanism(s) by which this organism binds to epithelial cells and to investigate whether attachment leads to internalization. In this study, we used an in vitro model system to demonstrate that certain *P. gingivalis* strains can bind to and invade human oral epithelial cells.

* Corresponding author.

† Present address: Department of Pharmacology, Nihon University School of Dentistry, 870-1 Sakaecho-Nishi, Matsudo Chiba, 271 Japan.

‡ Present address: Department of Oral Biology, School of Dentistry, Health Sciences Center, University of Washington, Seattle, WA 98195.

The *P. gingivalis* and *Escherichia coli* strains used in this study together with their origins are listed in Table 1. Strains from K. Klimpel originated from the collection of Caroline Attardo Genco, Morehouse School of Medicine, Atlanta, Ga. *P. gingivalis* strains were grown on blood agar plates supplemented with heme (0.5 μg/ml) and menadione (1 μg/ml) (BAPHK) for 48 h at 37°C in a Coy anaerobic chamber (80% N_2, 10% CO_2, 10% H_2). *E. coli* strains were grown aerobically on Luria-Bertani broth plates (supplemented with ampicillin at 25 μg/ml for the maintenance of pRI203) overnight at 37°C. KB (ATCC CCL17) is a human oral epithelial cell line obtained from the American Type Culture Collection, Rockville, Md. KB cells were grown at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn calf serum, gentamicin (50 μg/ml), 2 mM L-glutamine, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.3). For growth in 24-well tissue culture plates, wells were seeded with 10^5 cells and cells were grown to confluence (10^6 cells) for 48 h.

We used the assay described by Leong et al. (8) to screen *P. gingivalis* strains immobilized on filter membranes for their ability to bind KB cells. Bacteria were harvested from plates, washed twice, and resuspended at approximately 10^8 cells per ml in phosphate-buffered saline (PBS). Aliquots of suspensions (3 μl) were spotted onto Immobilon-P filters (Millipore Corp., Bedford, Mass.) prewetted in methanol and then PBS. Filters were air dried on 3MM paper (Whatman, Maidstone, United Kingdom) and rewetted in methanol and then PBS. Bacterium-bearing filters were transferred to 3MM paper soaked with 0.1% sodium dodecyl sulfate (SDS) in 10 mM Tris (pH 7.9) for 30 min at room temperature. This treatment enhanced bacterial binding to epithelial cells. For consistent binding, certain strains also required a similar soaking with lysozyme (1 mg/ml in 10 mM Tris [pH 7.9]–25 mM EDTA) for 30 min at room temperature prior to SDS exposure. Filters were dunked, colony side down, in 5% nonfat dry milk in PBS and incubated overnight at room temperature with shaking to prevent nonspecific binding of epithelial cells to the filter. The filter was washed three times in PBS (5 min with shaking at room temperature), reblocked for 1 h, and washed again. KB cells grown in flasks were washed twice with PBS and detached by incubation in 4.3 ml of PBS with 2 mM EDTA for 20 min at 37°C in 5% CO_2. $MgCl_2$ was added to 5 mM, and the cells were washed with

TABLE 1. *P. gingivalis* strains used and KB cell binding

Organism and strain	Source[a]	Comments	KB cell binding
P. gingivalis			
381	FDC	Type strain	+
131	FDC	Clinical isolate	+
431	FDC	Clinical isolate	+
151	FDC	Clinical isolate	+
451	FDC	Clinical isolate	+
33277	K. Klimpel	Type strain	+
HG66	K. Klimpel	Clinical isolate	+
HG405	K. Klimpel	Laboratory strain	+
A7436	K. Klimpel	Clinical isolate	+[b]
W50	K. Klimpel	Laboratory strain	−
W50/BE1	K. Klimpel	Avirulent variant of W50	−
E. coli			
MC1000/pRI203	R. Isberg	Positive control	+
MC1061	FDC	Negative control	−

[a] FDC, Forsyth Dental Center, Boston, Mass.; K. Klimpel, Colgate-Palmolive, Piscataway, N.J.; R. Isberg, Tufts University, Boston, Mass.
[b] A7436 bound KB cells after treatment with lysozyme, EDTA, and SDS (see text).

40 ml of PBS–5 mM $MgCl_2$ and then with PBS containing 1 mM $MgCl_2$–0.2 mM $CaCl_2$. Finally, KB cells were resuspended at 5×10^5 to 1×10^6 cells per ml in serum-free DMEM containing 20 mM HEPES (pH 7.0), 0.4% bovine serum albumin, 1 mM $MgCl_2$, and 0.2 mM $CaCl_2$. KB cells were added to the bacterium-bearing filters (at least 2×10^5 cells per cm^2 of filter were needed for an unequivocal result), and the filters were incubated for 90 min at 37°C in 5% CO_2. The filters were washed three times in PBS and then fixed with 1% electron-microscopy-grade glutaraldehyde in 100 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); pH 6.9]–50 mM KCl–4 mM $MgCl_2$ (0.15 ml of solution per cm^2 of filter) for 22 min at room temperature. Filters were again washed three times in PBS. Epithelial cells bound to bacteria were revealed by staining their alkaline phosphatase activity with the synthetic chromogenic substrates bromo-chloro-indolyl phosphate (50 μg/ml) and nitroblue tetrazolium (100 μg/ml) in 100 mM Tris (pH 9.5), 100 mM NaCl, and 5 mM $MgCl_2$. The reaction was for 30 min at room temperature in the dark, and it was stopped by immersing the filter in PBS with 2 mM EDTA. Figure 1a shows dark spots of alkaline-phosphatase-stained epithelial cells bound to *P. gingivalis* 33277, while Fig. 1b shows strain W50, which did not bind epithelial cells and was a uniform pale color, presumably because of the background level of bacterial alkaline phosphatase. Figure 2a is a scanning electron micrograph of a filter section showing KB cell binding by bacteria, while the filter section in Fig. 2b was from a nonbinding control strain. Table 1 lists strains which have been tested and their binding abilities. After SDS treatment, several *P. gingivalis* strains bound KB epithelial cells. Strain A7436 gave a variable reaction but always reacted positively if treated with lysozyme prior to exposure to SDS. In addition to strain W50, a derivative, W50/BE1, also consistently failed to bind to epithelial cells irrespective of pretreatment. The negative control was *E. coli* MC1061, which did not bind KB cells even after treatment with lysozyme plus SDS. The positive control was *E. coli* MC1000, which contained the cloned invasin gene from *Yersinia pseudotuberculosis* on the plasmid pRI203. This strain bound KB cells, but as with *P. gingivalis*, binding was enhanced after SDS treatment. Although lysis due to SDS cannot be ruled out, these results might also imply that the bacterial component involved in epithelial cell binding is localized close to the surface but is either masked or in an unreactive conformation in bacteria cultured under the conditions described. Indeed, all of the *P. gingivalis* strains used in this study possessed outer-surface-associated lipopolysaccharide (3a, 7), so SDS treatment may expose ligands by cell lysis or by inducing changes at the bacterial surface. Growth of *P. gingivalis* strains on blood or Trypticase soy plates or in Trypticase soy broth did not qualitatively alter the ability to bind epithelial cells; however, experiments under heme-limiting conditions have not yet been carried out. That *P. gingivalis* can bind KB cells in this assay is significant. The same protocol with HEp-2 cells was used to screen a range of bacterial pathogens for adhesins and invasins (8). Only invasive strains of *Y. pseudotuberculosis*, tissue culture-invasive strains of *Yersinia enterocolitica*, and *Borrelia burgdorferi* gave a consistent positive epithelial cell-binding signal. Obviously, the stringency of the assay precludes not only adhesin-mediated epithelial cell binding but also binding by invasins from other bacterial species which may use other invasion pathways. It has been suggested (4) that the high-affinity binding characteristic of this assay is necessary for bacterial ligands which must compete for multifunctional receptors on epithelial cells.

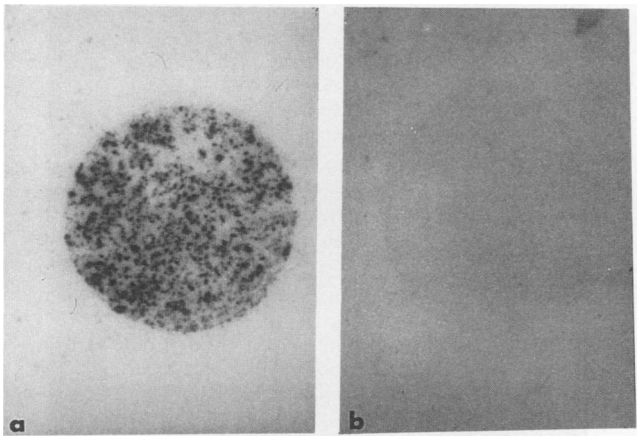

FIG. 1. *P. gingivalis*-mediated KB epithelial cell binding to Immobilon filters. (a) Alkaline-phosphatase-stained epithelial cells binding to strain 33277; (b) negative reaction with strain W50. Magnification, ×15.

The binding of bacteria to epithelial cells was quantitated by viable counting of *P. gingivalis* cells attached to KB monolayers. *P. gingivalis* 33277 and W50 were grown on BAPHK and washed and resuspended in PBS as described above. Bacteria were not pretreated for this assay. Bacterial suspensions were appropriately diluted (the range was from approximately 10^4 to 10^8 cells per ml) in DMEM containing 1 mM $MgCl_2$ and 0.2 mM $CaCl_2$. One milliliter of bacterial suspension was added to PBS-washed confluent KB monolayers (10^6 cells per well in a 24-well plate) and incubated at 37°C in 5% CO_2 for 2 h. After incubation, unattached bacteria were removed and the monolayers were washed three times with agitation in PBS. Epithelial cells were lysed in 1 ml of sterile distilled water per well and incubated for a further 10 min. Lysates were diluted for viable counting, plated on BAPHK, and incubated anaerobically at 37°C. The results obtained in the quantitative filter binding assay are

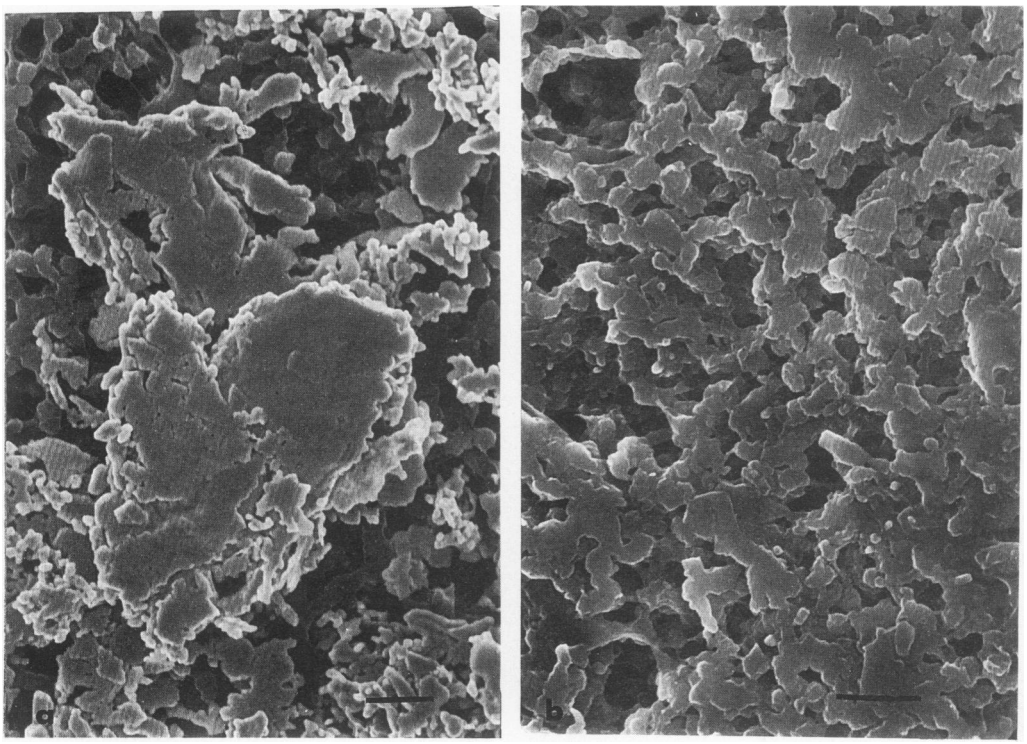

FIG. 2. Scanning electron microscopy of KB cell binding to Immobilon filters. (a) Region of filter corresponding to stained KB cells; (b) region of filter from negatively reacting strain which does not bind KB cells. Bars, 2 μM.

shown in Table 2. Most striking is the poor binding of *P. gingivalis* W50 compared with that of strain 33277, confirming the results of the qualitative filter binding assay. *P. gingivalis* 33277 adherence was measured over a range of multiplicities of infection, since one might expect high-specificity binding to epithelial cells at low bacterial cell densities and less-specific binding at higher bacterial densities. Interestingly, over a range of concentrations, a subset of approximately 10% of strain 33277 cells bound to the KB monolayer. This result suggests that the expression of the binding component is regulated; however, to date, we do not know whether this is controlled by growth phase or nutritional requirements. When higher concentrations of bacteria were added to monolayers, larger numbers were apparently able to bind (data not shown); however, this reflects the aggregation of bacteria to each other in addition to attach-

TABLE 2. Attachment of *P. gingivalis* strains to monolayers of KB cells

Strain	Bacteria/ml added	% Adhesion (mean ± SD)[a]
33277	5.9×10^4	13.5 ± 10.77
	1.1×10^5	9.7 ± 2.64
	1.3×10^6	10.5 ± 3.68
	1.7×10^7	11.2 ± 7.25
	1.5×10^8	8.1 ± 6.6
W50	4.0×10^6	0.001 ± 0
	4.4×10^7	0.005 ± 0.001
	4.3×10^8	0.024 ± 0.002

[a] Values represent the means plus or minus standard deviations of triplicate or quadruplicate samples and reflect similar results from several experiments.

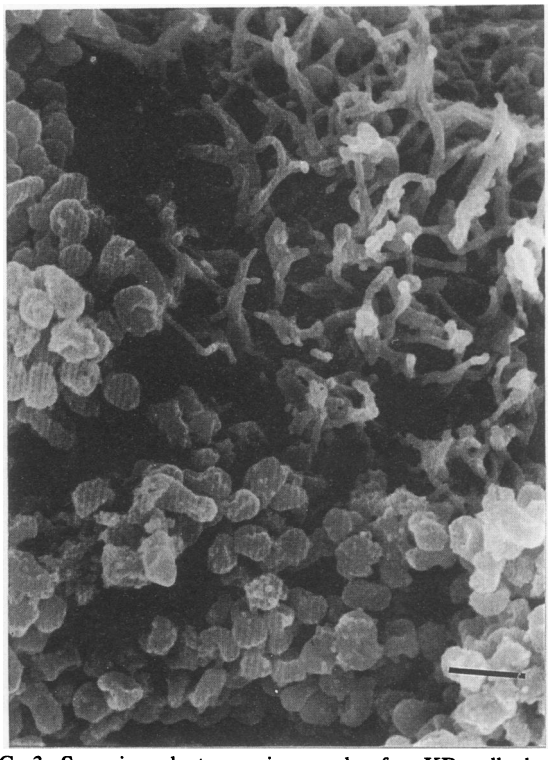

FIG. 3. Scanning electron micrograph of a KB cell showing microvilli with attached and autoaggregated *P. gingivalis* cells. Bar, 1 μM.

TABLE 3. Invasion of KB human oral epithelial cells by *P. gingivalis* strains

Strain	Bacteria/ml added	Bacteria/ml recovered after antibiotic treatment (mean ± SD)[a]
P. gingivalis 33277	6.3×10^6	$(4.3 \pm 3.3) \times 10^3$
	2.1×10^8	$(1.4 \pm 0.8) \times 10^5$
E. coli		
MC1000/pRI203	7.9×10^8	$(3.6 \pm 1.2) \times 10^5$
MC1061	7.4×10^8	$(2.4 \pm 1.6) \times 10^3$

[a] Values represent the means plus or minus standard deviations of triplicate or quadruplicate samples and reflect similar results from several experiments.

ment to KB cells, as illustrated by the scanning electron micrograph shown in Fig. 3. The quantitative assay differs from the filter binding assay in at least two respects. First, untreated bacteria attached to KB monolayers in the quantitative assay while bacteria were treated with SDS in the filter binding assay. Although we do not know if the same bacterial binding components are being tested, at least two strains behaved similarly in both assays. Second, serum was present during the quantitative assay. However, similar quantitative results were obtained after the addition of suspensions of radiolabeled *P. gingivalis* strains in buffer to KB monolayers (12). Autoaggregation of several *P. gingivalis* strains was also observed by these workers.

We modified a quantitative assay (3) to show that cells of *P. gingivalis* 33277 were internalized by KB cells. Bacteria were harvested, washed, and resuspended as described above in complete DMEM without antibiotic. *E. coli* MC1000/pRI203 and MC1061 were the positive and negative controls, respectively, for invasion. Confluent KB monolayers in 24-well plates were washed three times with PBS and infected with 1 ml of bacterial suspensions. Tissue culture plates were centrifuged at 1,000 rpm for 10 min at 4°C to enhance contact between KB cells and bacteria. Infected monolayers were incubated for 4 h at 37°C in a 5% CO_2 incubator. Unattached bacteria were removed, and the monolayers were washed three times with PBS. External, adherent cells of *P. gingivalis* 33277 were killed by incubating infected monolayers in 1 ml of DMEM containing 100 μg of the bactericidal antibiotic trospectomycin per ml (a gift from The Upjohn Co., Kalamazoo, Mich.) for 1 h at 37°C in 5% CO_2. Adherent cells of *E. coli* were killed by similar incubations with 100 μg of gentamicin per ml. Incubations of bacteria alone in the antibiotic solutions under the same conditions reduced viability by 10^6. Monolayers were washed three times in PBS and lysed in distilled water, and the lysates were diluted for viable counting of bacteria as described above. Table 3 shows the number of bacteria of *P. gingivalis* 33277 and the *E. coli* control strains which survived the antibiotic treatments. The positive control strain *E. coli* MC1000/pRI203 invaded KB cells with low efficiency (i.e., the number of bacteria surviving antibiotic treatment as a percentage of the bacteria added) compared with published values for invasion of HEp-2 cells (5, 13). This discrepancy may reflect a number of variables, of which differences between KB and HEp-2 cell surface receptors and the experimental protocols are the most likely. The invasion efficiency of *P. gingivalis* 33277, at both cell concentrations, was similar to that of the positive control. Such values were not surprising given that attachment assay results and transmission electron micrographs (see below) indicate that approximately 10% of the *P. gingivalis* cells added invaded a similarly small subset of KB cells in the monolayers. The actual numbers of recovered bacteria were similar to those reported by Sandros et al. (12) in studies of KB cell invasion by several *P. gingivalis* strains. The invasion efficiency of the negative control strain MC1061 was at least 60-fold less than that of the experimental strains.

For electron microscopy, after a 4-h bacterial infection, monolayers were flooded for 4 h with half-strength Karnovsky's fixative (2% glutaraldehyde–2% formaldehyde in 0.1 M cacodylate buffer [pH 7.2]) and then washed for 2 h in several volumes of 0.1 M cacodylate and postfixed for 4 h in 2% osmium tetroxide in 0.1 M collidine buffer with 0.1% ruthenium red. After the monolayers were rinsed with water, dehydration was initiated with 50% ethanol saturated with uranyl acetate and continued in increasing ethanol concentrations without uranyl acetate. Monolayers in 100% ethanol were treated with acetone, and ribbons of cells were detached and embedded for propylene oxide treatment. Thin sections were stained with lead citrate and examined with a JEOL 1200 EX transmission electron microscope. Figures 4a and b show cells of *P. gingivalis* 33277 attached to and inside KB epithelial cells. Ruthenium red, to stain glycoprotein, was added after monolayer fixation. Since this stain does not penetrate the epithelial cell membrane, only extracellular bacteria bind the dye and appear coated with electron-dense material. Figure 4a shows a section of a KB cell in which at least 4 intracellular bacteria were visible, although it was not clear whether or not these bacteria were enclosed within a vacuole. Externally attached bacteria appeared to be in direct contact with the KB cell membrane. Figure 4b shows a section of an epithelial cell with 2 internalized bacteria, possibly surrounded by a membrane. Membranes surrounding 3 ruthenium-red-coated bacteria which may be in the process of being internalized are visible. Again, external bacteria were attached to the KB cell membrane and to microvilli. By viewing several fields, it was estimated that approximately 10% of the epithelial cells contained bacteria. This may reflect the specific attachment by a subset of the *P. gingivalis* 33277 cells as indicated from the quantitative assays and inefficient invasion by this strain or even the species. However, we know little as yet of the growth conditions which regulate bacterial binding or invasion of epithelial cells, and it may be that low levels of a bacterial ligand, or even of an exogenous factor, limited invasion. Alternatively, although we used epithelial cells of oral origin in the assay, the number of *P. gingivalis* receptors they possess may be small compared with that of the gingival epithelial cells with which this organism interacts in vivo. In separate infections, *P. gingivalis* 381 was also seen within KB cells (not shown). Figure 4c shows that cells of *P. gingivalis* W50 were not in close contact with epithelial cell membranes, supporting the results of the epithelial cell binding assays. KB cell invasion by *E. coli* MC1000/pRI203 is shown in Fig. 4d.

We have demonstrated that certain *P. gingivalis* strains can bind to and invade human oral epithelial cells in vitro. Currently, we are establishing the bacterial growth and assay conditions which will optimize invasion efficiency. We do not yet know the relationship between the attachment and invasion activities; however, cloning of the genes for these functions will unequivocally prove their identity and relationship.

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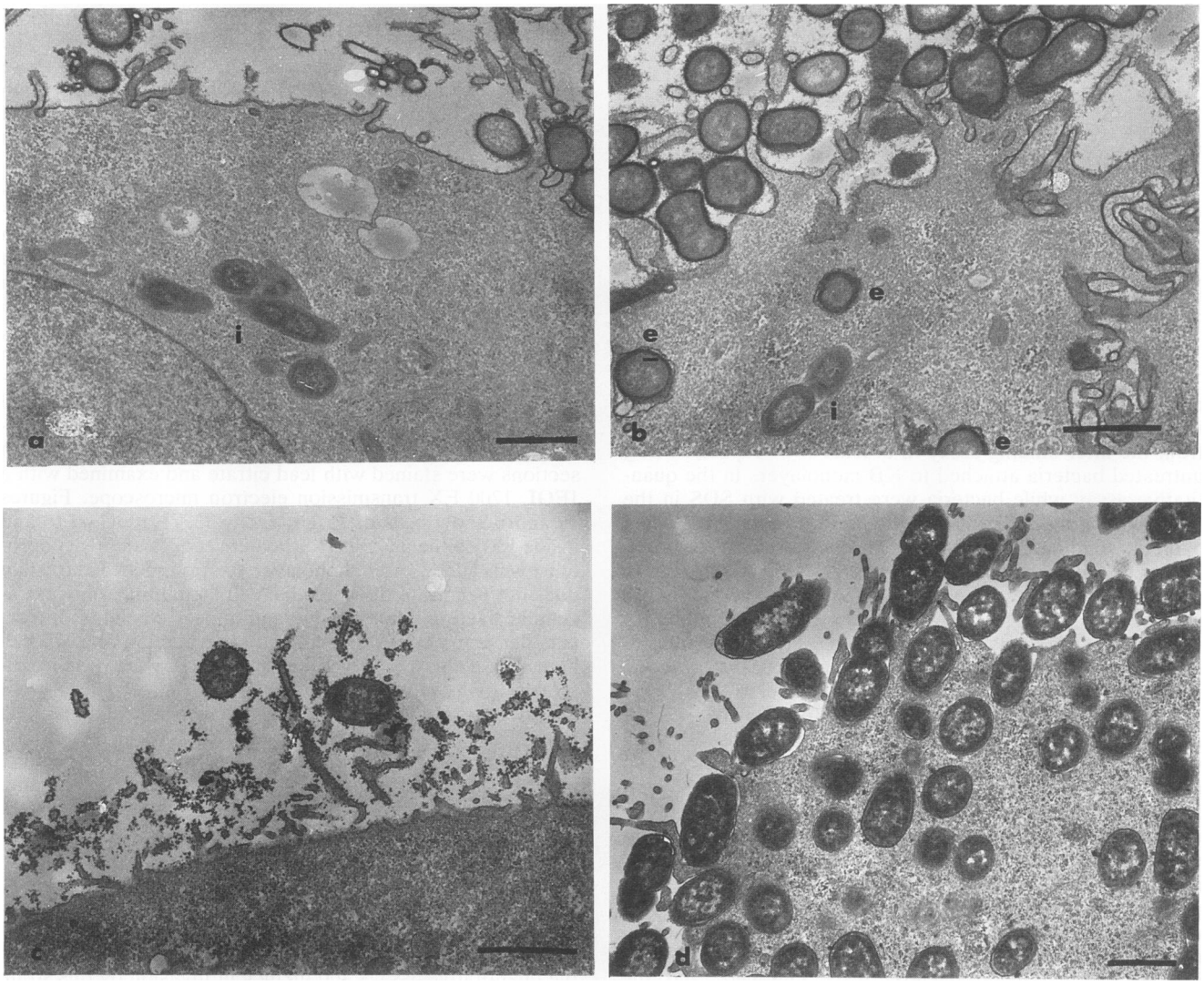

FIG. 4. Transmission electron micrographs of KB cells infected with *P. gingivalis* and *E. coli* strains. A 4-h infection at a multiplicity of infection of approximately 100:1 was followed by fixing and ruthenium red staining. (a and b) Strain 33277. Abbreviations: i, internalized bacteria; e, extracellular bacteria with electron-dense ruthenium red coating. (c) Strain W50. (d) *E. coli* MC1000/pRI203. Bars, 1 μM.

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