C.K. Mantri¹, C. Chen^{2*}, X. Dong³,
J.S. Goodwin⁴, and H. Xie^{1*}

¹Department of Oral Biology, School of Dentistry, Meharry Medical College, Nashville, TN, USA; ²Department of Surgery, Duke University Medical Center, Durham, NC, USA; ³Department of Microbiology and Immunology, Meharry Medical College, Nashville, TN, USA; and ⁴Department of Biochemistry and Cancer Biology, Meharry Medical College, Nashville, TN, USA; *corresponding authors, hxie@mmc.edu, chc@duke.edu

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ABSTRACT

HIV-1 relies on the host's cell machinery to establish a successful infection. Surface receptors, such as CD4, CCR5, and CXCR4 of T cells and macrophages, are essential for membrane fusion of HIV-1, an initiate step in viral entry. However, it is not well defined how HIV-1 infects CD4-negative mucosal epithelial cells. Here we show that there is a specific interaction between HIV-1 and an invasive oral bacterium, *Porphyromonas gingivalis*. We found that HIV-1 was trapped on the bacterial surface, which led to internalization of HIV-1 virions as the bacteria invaded CD4-negative epithelial cells. Both bacterial and viral DNA was detected in HeLa and TERT-2 cells exposed to the HIV-1-*P. gingivalis* complexes 2 hr after the initial infection but not in cells exposed to HIV-1 alone. Moreover, epithelial cell entry of HIV-1 was positively correlated with invasive activity of the *P. gingivalis* strains tested, even when the binding affinities of HIV-1 to these strains were similar. Finally, it was demonstrated that the viral DNA was integrated into the genome of the host epithelial cells. These results reveal a receptor-independent HIV-1 entry into epithelial cells, which may be relevant in HIV transmission in other mucosal epithelia where complex microbial communities can be found.

KEY WORDS: *P. gingivalis* 33277, HeLa cells, TERT-2 cells, bacterial invasion, CXCR4, CCR5.

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Porphyromonas gingivalis-mediated Epithelial Cell Entry of HIV-1

INTRODUCTION

More than 34 million people around the world are living with HIV-1. According to recent Center for Disease Control and Prevention reports. Approximately 90% of all HIV-1 transmissions occur mucosally (Kresina and Mathieson, 1999). In general, epithelial surfaces are the first line of defense against pathogens such as bacteria and viruses. However, HIV-1 appears to be able to penetrate across a mono- or multilayered epithelium, even in the absence of breaches and lesions (Hladik and Hope, 2009). Several mechanisms have been implicated to explain how HIV-1 overcomes the mucosal barrier and establishes an infection (Morrow *et al.*, 2008; Southern, 2013). First, mucosal dendritic cells appear to be important in HIV-1 entry through the epithelium, as cell-free or cell-associated HIV-1 may be captured by dendrites extending through the epithelial layers. Second, HIV-1 may be able to cross the epithelial layer by trafficking through intracellular compartments or by reaching the underlying tissues of the epithelial layer via transcytosis, which transfers HIV-1 to target cells such as dendritic cells, T cells, and macrophages. Previous studies suggest that some of the surface molecules, including HIV-1 coreceptors CXCR4 and CCR5, are likely responsible for the HIV-1 entry into epithelial cells (Bomsel, 1997; Alfsen and Bomsel, 2002; Bobardt *et al.*, 2007). For instance, gp340 on the cell surfaces of female genital tract epithelial cells (Stoddard *et al.*, 2009), DEC-205 on renal epithelial cells (Hatsukari *et al.*, 2007), and CCR5 on oral keratinocytes (Giacaman *et al.*, 2007) may mediate HIV-1 entry into these cells.

Previously, we found that surface extracts of *Porphyromonas gingivalis* inhibit HIV-1 infection of CD4-positive cells (Xie *et al.*, 2006). A surface protein of *P. gingivalis*, the binding domain of rg-gingipain-1 (HGP44), was identified as the molecule that interacts with HIV-1 gp120. This interaction was implicated to be the mechanism responsible for the ability of *P. gingivalis* to block HIV-1 from entering CD4 positive cells. *P. gingivalis* is a gram-negative anaerobic bacterium associated with adult periodontitis and is more prevalent in both supra- and subgingival dental plaque samples from periodontitis subjects compared to levels seen in the oral cavities of healthy subjects (Ximenez-Fyvie *et al.*, 2000; Colombo *et al.*, 2006). One important feature of *P. gingivalis* is its ability to invade epithelial cells, including primary gingival epithelial cells, and epithelial cell lines such as HeLa cells (Lamont *et al.*, 1995; Tsuda *et al.*, 2008). *P. gingivalis* binds to epithelial cells through adhesive molecules (*e.g.*, fimbriae) that directly engage $\alpha v \beta 3$ and $\alpha 5 \beta 1$ integrins on the surface of oral epithelial cells (Tribble and Lamont, 2010). This leads to rearrangement of the host cytoskeleton and allows

bacterial entry. Based on the affinity of HIV-1 to *P. gingivalis* surface proteins, we proposed that coinfection of HIV-1 and *P. gingivalis* may be a dangerous liaison for oral transmission of HIV-1.

MATERIALS & METHODS

Bacterial Strains and Growth Conditions

P. gingivalis strains—including wild-type 33277 with long fimbriae, its fimbrial-deficient mutant (FAE) with an insertional mutation in the *fimA* gene, and wild-type W83, which expresses neither major nor minor fimbriae—were grown from frozen stocks in trypticase soy broth or on trypticase soy broth blood agar plates supplemented with yeast extract (1 mg/mL), hemin (5 µg/mL), and menadione (1 µg/mL) and incubated at 37°C in an anaerobic chamber (85% N₂, 10% H₂, 5% CO₂). Antibiotics were used when appropriate, at the following concentrations: gentamicin (100 µg/mL) and erythromycin (5 µg/mL). *Streptococcus gordonii* G9B was grown in trypticase peptone broth supplemented with 0.5% glucose at 37°C under aerobic conditions. *Escherichia coli* DH5α was grown in Luria broth at 37°C under aerobic conditions. Antibiotics and chemical agents were purchased from Sigma (St. Louis, MO, USA) unless otherwise indicated.

Cell Culture and HIV Virus Production

HeLa and 293T (human embryonic kidney cells) cell lines were obtained from ATCC, and TERT-2 cells (immortalized oral keratinocytes) were kindly provided by Dr. Bingdong Liu (Meharry Medical College, Nashville, TN, USA). HeLa and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL of penicillin, and 100 µg/mL of streptomycin at 37°C in 5% CO₂. TERT-2 cells were cultured in keratinocyte-SFM (Invitrogen) supplemented with 25 µg/mL of bovine pituitary extract, 100 U/mL of penicillin, 100 µg/mL of streptomycin, 0.2 ng/mL of epidermal growth factor, and 0.3mM CaCl₂. X4-tropic HIV-1_{NL4-3} and R5-tropic HIV-1_{YU2} virions were produced by transfection of 293T cells with pNL4-3 or pYU2 (AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA), respectively, via a FuGENE 6 reagent (Promega, Madison, WI, USA). The viral supernatants were collected at 48-hr posttransfection and stored in aliquots (–80°C); p24 antigens in the viral stocks were quantified through an enzyme-linked immunosorbent assay (ELISA) kit from PerkinElmer Life Sciences (Wellesley, MA, USA) (Xie *et al.*, 2006).

Bacteria and HIV-1 Interaction

Bacteria were grown into midlog phase and harvested, washed, and resuspended in phosphate-buffered saline (PBS). Bacteria (10⁷) were mixed with HIV-1 virions (250 ng of p24) in 500 µL of DMEM and incubated at room temperature for 30 min. Unbound virions were removed by centrifugation. The bacteria-virions complexes were washed with PBS and resuspended in

DMEM. The binding affinity of HIV-1 to each bacterial strain was determined via a p24 ELISA.

Coinfection of Epithelial Cells with *P. gingivalis* and HIV-1

HeLa and TERT-2 cells were placed in 6-well tissue culture plates (Celltreat, Shirley, MA, USA) and grown overnight to 50% to 60% confluence. The bacteria-HIV-1 complexes or cell-free HIV-1 was added to each well seeded with HeLa or TERT-2 cells. The cells were incubated in DMEM in a CO₂ incubator for 1 hr. Cells were then washed with DMEM and incubated with gentamicin (300 µg/mL) and metronidazole (200 µg/mL) to eliminate extracellular bacteria for another 1, 2, 4, or 23 hr. Cells were collected with trypsin treatment and washed with PBS before DNA extractions were performed.

DNA Isolation and Quantitative Polymerase Chain Reaction

DNA was extracted from epithelial cells infected with or without bacteria, *P. gingivalis*-HIV-1 complexes, and cell-free HIV-1 with an Easy-DNA kit (Invitrogen). Intracellular *P. gingivalis* was determined through quantitative polymerase chain reaction (PCR) analyses with bacterial *16S rRNA* primers and reverse-transcript HIV-1 DNA with *gag* primers (Appendix Table). Quantitative PCR analysis was performed with an iQSYBR Green Supermix (Bio-Rad, Hercules, CA, USA) on the iCycler MyiQ Real-Time PCR detection system (Bio-Rad) according to the manufacturer's instructions. Genomic DNA of epithelial cells without exposure to bacteria or HIV-1 was used as a negative control.

Confocal Immunofluorescence

HeLa cells were grown overnight on glass-bottom microwell dishes (MatTek, Ashland, MA, USA) and infected with *P. gingivalis* 33277, green fluorescent protein-tagged NL4-3, or 33277–NL4-3 complexes for 30 min in 5% CO₂. The infected cells were fixed with 3.8% formaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 5% bovine serum albumin. *P. gingivalis* was stained with rabbit polyclonal anti-FimA antiserum, followed by goat anti-rabbit Alexa 546-conjugated antibodies (Invitrogen). Confocal images were acquired with a Nikon A1R confocal microscope, and data analysis was performed with EZ-C1 and NIS-Elements AR software.

Quantitation of Integrated Provirus

Copies of integrated provirus were determined through an Alu-long terminal repeat (LTR)-based real-time nested PCR assay (Brussel and Sonigo, 2003). Two human genomic Alu forward primers and an HIV-1 LTR reverse primer extended with a lambda phage-specific heel sequence at the 5' end of the oligonucleotide (L-M667) were used to enrich host genomic DNA with downstream proviral DNA based on PCR. The integrated HIV-1 provirus was then quantified via an iQ Supermix kit (Bio-Rad) with a reaction solution including lambda-specific primer

Figure 1. Interaction of HIV-1 and *Porphyromonas gingivalis* and *Streptococcus gordonii*. (A) *P. gingivalis* strains 33277, FAE, W83, as well as *S. gordonii* G9B and *E. coli* DH5 α , were incubated with NL4.3 or YU2 (p24, 250 ng) for 30 min and the bacteria/HIV-1 complexes collected by centrifugation. Quantitation of HIV-1 bound to bacterial surfaces was conducted by a p24 assay. Asterisk (*) indicates a statistically significant difference in p24 levels for comparison of p24 reading of phosphate-buffered saline (PBS) with that of bacteria incubated with HIV-1 (*t* test; *p* < .05). (B) *P. gingivalis* 33277 cells were exposed to different doses of NL4.3 for 30 min. (C) Bacterial cells were mixed with green fluorescent protein (GFP)-tagged HIV-1 NL4.3 for 30 min, and free virions were removed by washing with PBS. *P. gingivalis* cells were identified by a rabbit anti-FimA polyclonal antibody, and *S. gordonii* G9B was identified by a rabbit anti-SpB polyclonal antibody. Both were visualized by TRITC-conjugated AffiniPure goat anti-rabbit IgG antibody (red).

(Lambda T), LTR primer (AA55M), Taqman probe (AE995) (Appendix Table), and the PCR products from the first-round PCR. A curve of the integrated-HIV copy number standard was prepared from DNA isolated from ACH-2 cells, which harbor a known copy number of integrated provirus (O'Doherty et al., 2002). The signal potentially contributed from unintegrated HIV-1 DNA was subtracted from the total signal by including a one-way preamplification via an LTR primer. Amplification of the human *gapdh* gene was used for normalization of copies of HIV-1 provirus.

Enzyme-Linked Immunosorbent Assay

Epithelial cells (3×10^4 /well) were grown in a 96-well plate for 16 hr and then infected with *P. gingivalis* 33277 (3×10^6), cell-free HIV-1 (NL4.3 or YU2, p24 8 μ g), or 33277-HIV-1 complexes for 1 hr. Unbound bacteria cells and virions were removed and incubated in a complete medium for another 1 hr. Cells were then washed, fixed with 3.8% formaldehyde, and blocked with 3% BSA. Anti-CCR5 monoclonal antibodies (MAb hCCR5, 45531.111) and anti-CXCR4 monoclonal antibodies (MAb hCXCR4, 44717.111) (AIDS Research and Reference Reagent Program, 1:1,000) were then applied to the plates and incubated for 3 hr. The plates were washed with PBS and incubated with horseradish peroxidase-conjugated antibodies against rabbit IgG (1:1,000, Invitrogen). Peroxide substrate (Sigma) was added to each well after being washed 5 times with

PBS. The reaction was stopped by the addition of 100 μ L of 1 N H₂SO₄. The results were read (450 nm) on a Benchmark Plus microplate reader (Bio-Rad).

Statistical Analyses

A Student's *t* test was used to determine statistical significance of the differences in the rates of detection of bacteria or HIV-1 and in gene expression profiles. A *p* value < .05 was considered significant. Values are shown $\pm$ SD.

RESULTS

Interaction of *P. gingivalis* and HIV-1

To determine whether there is a specific interaction between HIV-1 and *P. gingivalis*, we examined viral binding to different *P. gingivalis* strains. Since long fimbriae appear to be required for the binding and invasive abilities of *P. gingivalis* (Weinberg et al., 1997; Sojar et al., 1999), 3 strains of *P. gingivalis* were tested, including 1 fimbriated strain (33277) and 2 afimbriated strains (FAE and W83). *S. gordonii* G9B, a dominant oral commensal bacterium, and *E. coli* DH5 α were used as controls. Bacterial cells were incubated with HIV-1_{NL4.3} (X4 type) or HIV-1_{YU2} (R5 type), respectively. The relative amount of each HIV-1 strain bound to bacteria was determined through a p24 ELISA. The enhanced viral and bacterial interactions were found among *P. gingivalis* 33277, FAE, and W83 cells with NL4-3 and between *P. gingivalis* 33277 and FAE with YU2, while there was no significant binding of HIV-1 to *S. gordonii* and *E. coli* (Figure 1A). These results demonstrate that HIV-1 specifically binds to *P. gingivalis* cells and that the major fimbriae of the organism are not required for the bacteria-virus interaction. We also demonstrated a dose-dependent manner of HIV-1_{NL4.3} binding to *P. gingivalis* 33277 when the bacterial cells were exposed to different concentrations of virions ranged from 1 to 250 ng of p24 (Figure 1B).

The binding of HIV-1_{NL4.3} virions tagged with Vpr-GFP (green fluorescent protein) (Schaeffer et al., 2001) to *P. gingivalis* 33277 was visualized under an inverted confocal microscope (Figure 1C). Consistent with p24 ELISA results, virions (in green) were observed on the surfaces of *P. gingivalis* cells but not on *S. gordonii*. The results provide strong evidence to support our earlier suggestion of a high affinity between *P. gingivalis* and HIV-1.

Promoting HIV-1 Entry of Epithelial Cell by *P. gingivalis*

To test that binding of HIV-1 to *P. gingivalis* leads to viral entry into epithelial cells through a receptor-independent pathway, HeLa and TERT-2 cells were infected with the bacteria-associated

virions or cell-free HIV-1 for 1 hr. Using quantitative PCR analyses, we detected intracellular *P. gingivalis* with the bacterial *16S rRNA* primers and the reverse-transcribed HIV-1 DNA, an indicator of cell entry of HIV-1, with *gag* primers (Appendix Table). The results showed that the fimbriae-deficient strains, FAE and W83, had a significantly lower ability to enter HeLa cells when compared with 33277, supporting the notion that long fimbriae play an important role in *P. gingivalis* invasion (Figure 2A). HIV-1 *gag* DNA was detected in HeLa cells 2 hr after infection with *P. gingivalis*–HIV-1 complexes, and the rates of *gag* DNA detection corresponded to invasive abilities of the *P. gingivalis* strains (Figure 2B, C). No significant amounts of *gag* DNA were found in cells infected with cell-free HIV-1 or cells incubated with *S. gordonii* and HIV-1. When interacted with *P. gingivalis*, both HIV-1_{NL4.3} and HIV-1_{YU2} showed a comparable ability to infect epithelial cells, indicating that HIV-1 coreceptors CXCR4 and CCR5 are not necessary for this bacteria-mediated HIV-1 entry since NL4.3 and YU2 use CXCR4 and CCR5 for cell entry, respectively. Viral DNA and *P. gingivalis* DNA reached their highest levels 3 hr after the initial infection with *P. gingivalis*–NL4.3 complexes. The detected levels of both HIV-1 and *P. gingivalis* decreased over the next 21 hr (Figure 2D). Presumably, most viral and bacterial DNA was degraded in host cells during this period. Similar results were observed when TERT-2 cells were infected with *P. gingivalis*–HIV-1 complexes (Appendix Figure), which further supports an invasive bacterial-dependent entry of HIV-1 into epithelial cells.

P. gingivalis–mediated HIV-1 entry of epithelial cells was also confirmed through confocal microscopy. After being infected with *P. gingivalis* 33277–Vpr-GFP-labeled HIV-1_{NL4.3} complexes for 30 min, HeLa cells were analyzed with confocal microscopy and LSM software, which calculates measurements via x-y-z pixel dimensions to ensure that both *P. gingivalis* and HIV-1 are within the epithelial cells rather than on the cell surface. HIV-1_{NL4.3} was detected within HeLa cells as an intracellular punctuate distribution pattern characterized by the colocalization with *P. gingivalis* cells (Figure 3), suggesting that the invasive ability of *P. gingivalis* was not affected by HIV-1

Figure 2. *Porphyromonas gingivalis* promotes HIV-1 entry into HeLa cells. **(A)** Comparison of invasive ability of oral bacteria into HeLa cells 3 hr after the initial infection. **(B)** Comparison of the role of various bacteria in HIV-1_{NL4.3} entry of HeLa cells and **(C)** in HIV-1_{YU2} entry 3 hr after the initial infection. **(D)** The corresponding of bacterial *16S rRNA* and HIV-1_{NL4.3} *gag* DNA detected during a 24-hr period after the initial infection. Each bar represents the relative fold increase in *P. gingivalis* *16S rRNA* or HIV-1_{NL4.3} DNA detected in HeLa cells compared to its detection levels in noninfected cells (as 1 unit). Error bars represent SD ($n = 3$). An asterisk indicates a statistically significant difference in host cell levels with and without infection (t test; $p < .05$).

Figure 3. Visualization of intracellular HIV-1_{NL4.3} and *Porphyromonas gingivalis* 33277 in HeLa cells with a confocal microscopy. HeLa cells were grown in a glass bottom dish (MafTek, Boston) for 16 hr and infected with *P. gingivalis*–NL4.3 complexes for 30 min. Intracellular *P. gingivalis* cells and HIV-1_{NL4.3} were visualized under a confocal microscope (Nikon A1R). **(A)** Visualization of green fluorescent protein-tagged NL4.3 (green). **(B)** *P. gingivalis* cells were visualized by Alexa Fluor 546-conjugated anti-rabbit IgG secondary antibody (red). **(C)** Infected cells presented by differential interference contrast images. The dashed line indicates the boundary around an infected HeLa cell containing colocalization of both *P. gingivalis* and NL4.3.

binding and that at least some of the HIV-1 was still associated with bacterial cells 30 min after initiation of infection. In contrast, internalization of cell-free NL4.3 was not observed in HeLa cells (data not shown). Previous studies reported that *P. gingivalis* can be found in the cytoplasm or contained within membrane-bound vacuoles (Sandros *et al.*, 1993; Njoroge *et al.*,

Figure 4. Integration of HIV-1 into the genome of HeLa cells. HeLa cells were infected with cell-free NL4-3 or *Porphyromonas gingivalis* 33277-associated NL4-3 for 1 hr. The infected HeLa cells were incubated for another 2 hr after the unbound bacteria and virions were removed. Quantitation of integrated provirus was determined via an Alu-long terminal repeat-based real-time nested polymerase chain reaction assay. Each bar represents a mean of copies of integrated provirus per 10⁶ cells $\pm$ SD. The results were obtained from 3 independent experiments. An asterisk indicates a statistically significant difference in provirus copies in the cells infected with cell-free NL4.3 versus cells infected with *P. gingivalis*-NL4.3 complexes (*t* test; *p* < .05).

1997; Houalet-Jeanne *et al.*, 2001). Our results imply that *P. gingivalis*-NL4.3 complexes are unlikely to be constrained by membranous vacuoles, since reverse transcription of HIV-1 occurred at a relative short time after entry into epithelial cells.

To determine if HIV-1 DNA integrates into the genome of epithelial cells, an LTR-based real-time nested PCR assay was used (Brussel and Sonigo, 2003). Our results demonstrate that integrated provirus was found in HeLa cells 2 hr after the initial infection with *P. gingivalis*-NL4.3 complexes, whereas only a few copies of integrated provirus were detected in the cells infected with NL4.3 alone (Figure 4). This result is consistent with a previous report indicating that integrated HIV-1 DNA was detected in oral epithelial cells within 3 hr postinoculation of HIV-1_{NL4.3} (Vacharaksa *et al.*, 2008). Although we were not able to demonstrate that HIV-1-infected epithelial cells transfer or release infectious virions (data not shown), it cannot be ruled out at the present time whether infected cells may be activated and produce infectious virions under certain conditions, such as those under periodontal disease (Imai *et al.*, 2009).

Expression of HIV-1 Coreceptors on Epithelial Cells

P. gingivalis-promoted HIV-1 entry of epithelial cells has been reported (Giacaman *et al.*, 2007; Giacaman *et al.*, 2008). These investigators observed a selective upregulation of CCR5 in TERT-2 cells after pretreatment with *P. gingivalis* for 3 hr, as

well as an increased infection of the pretreated epithelial cells with R5-type, but not X4-type, HIV-1. To test whether expression of HIV-1 coreceptors CXCR4 and CCR5 were induced on the surfaces of HeLa and TERT-2 cells when examined 3 hr after infection under our experimental conditions, these cells were exposed to cell-free HIV-1, bacterial-associated HIV-1, or *P. gingivalis* for 1 hr and incubated for another 2 hr after the exposure. Expression levels of coreceptors were determined with an ELISA. As shown in Figure 5, expression of both HIV-1 coreceptors was not significantly upregulated upon exposure to cell-free HIV-1, *P. gingivalis*, or *P. gingivalis*-HIV-1 complexes compared to that found on unexposed cells. These results further indicate that the observed HIV-1 entry into epithelial cells did not result from an up-regulated expression of CCR5 and/or CXCR4, since *gag* DNA was detectable in HeLa and TERT-2 cells 2 hr after exposure to *P. gingivalis*-HIV-1 complexes.

DISCUSSION

The studies presented here are built on our previous work on the identification of a specific interaction between the adhesin domain (HGP44) of gingipains and HIV-1 gp120. We further demonstrate that HIV-1 virions selectively bind to the surfaces of *P. gingivalis* strains regardless of their expression of fimbriae. One exception was that HIV-1_{YU2} did not bind well to *P. gingivalis* W83, which may be due to the diversity in either HIV-1 gp120 or the binding domain of Arg-gingipain of *P. gingivalis* strains. In fact, considerable variability in genes encoding gingipains has been reported in 33277 vs. W83 (Mikolajczyk-Pawlinska *et al.*, 1998). Sequencing diversity is even more prominent in the HIV-1 gene due to error-prone reverse transcriptase, especially in the *env* genes. The most striking changes in diversity were found in HIV-1 gp41 and gp120 (Araujo and Almeida, 2013). Therefore, sequence diversity may result in differential affinities between gp120s and the binding domain of gingipains.

In this study, we also provide several lines of evidence to support *P. gingivalis* invasive activity-dependent HIV-1 entry into CD4-positive cells. First, the fimbriated *P. gingivalis* strain promoted significantly more HIV-1 entry than the afimbriated strains, although HIV-1 bound to these *P. gingivalis* strains equally well. Second, both NL4-3 and YU-2 virions were internalized into epithelial cells after being bound on *P. gingivalis* cells regardless of their tropism of coreceptors. Finally, we found that *P. gingivalis*-associated HIV-1 was able to enter both HeLa and TERT-2 cells at a similar level of efficiency, even though it was reported that TERT-2 cells express less CXCR4 than HeLa cells (Giacaman *et al.*, 2007). Interestingly, Giacaman *et al.* (2008) also reported that pretreated TERT-2 cells were more susceptible to R5-tropic HIV-1, likely due to upregulation of CCR5. These findings further underscore the significant role of *P. gingivalis* in oral transmission of HIV-1. In fact, under certain circumstances, HIV and *P. gingivalis* can coexist in the mouth of HIV-seropositive patients with periodontitis. The results from independent studies indicate that periodontopathogens, including *P. gingivalis*, were detected more frequently and were found at higher levels in HIV-infected patients than in

Figure 5. Expression of CXCR4 and CCR5 coreceptors on the surfaces of HeLa and TERT-2 cells. Following exposure to *Porphyromonas gingivalis* 33277, HIV-1, or *P. gingivalis*–HIV-1 complexes, the expressions of coreceptors were determined via an ELISA. (A) Anti-hCXCR4 and (B) anti-hCCR5 were applied to wells coated with HeLa or TERT-2 cells. The cells were then incubated with horseradish peroxidase–conjugated antibodies against mouse IgG. Each bar represents the relative expression of HIV-1 coreceptor on the surface of epithelial cells infected with the bacteria and/or HIV-1 compared to that in the cells noninfected (as 1 unit) ($n = 3$). Student's t test was used to determine statistical significance of the differences in the expression profiles.

noninfected control subjects with similar periodontal status (Scully *et al.*, 1999; Mellanen *et al.*, 2001). The efficiency of *P. gingivalis* invasion into epithelial cells is not conclusive, which ranged from 0.15% to 12% for *P. gingivalis* 33277 (Njoroge *et al.*, 1997; Weinberg *et al.*, 1997). A clinical study showed, with confocal microscopy, that *P. gingivalis* was detected within buccal epithelial cells taken directly from the mouths of healthy individuals (Rudney *et al.*, 2005). Therefore, it is conceivable that *P. gingivalis* could serve as a vehicle for oral mucosa transmission of HIV-1. It is also worthy to note that we showed, via confocal microscopy, a perinuclear distribution of *P. gingivalis* and HIV-1 (Appendix Figure), which is consistent with the earlier observation of *P. gingivalis* invasion (Yilmaz *et al.*, 2006). It is speculated that this apparent intracellular movement of *P. gingivalis* may result from actin rearrangement, since colocalization of *P. gingivalis* and actin was found perinuclearly.

In summary, *P. gingivalis*–mediated HIV-1 entry into CD4-negative epithelial cells defines a specific mechanism that contributes to HIV-1 infection and transmission of mucosa mediated by invasive bacteria. It may be relevant to other mucosal surfaces as well, such as those in intestinal, rectal, and vaginal sites, where mixed-species flora exist. Studying bacteria-mediated HIV-1 infection may further our understanding of HIV-1 pathogenesis, with an emphasis on the mucosal infection of HIV-1. It will be interesting in the future to determine if internalized HIV-1 in these epithelial cells establishes a latent or productive infection.

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