The predominant mode of human immunodeficiency virus (HIV) transmission is by sexual contact through broken mucosal or epidermal epithelia. It is rather clear that oral transmission of HIV is a rare event relative to vaginal and rectal transmission (3, 32). It has been reported that fewer infectious HIV particles are in saliva than in other body fluids, such as blood, semen, and vaginal and cervical secretions (25). However, hyperexcretion of HIV type 1 (HIV-1) RNA in saliva has been reported previously (27). It appears that 87% of HIV-infected patients had plasma titers exceeding those in saliva, and 7% of the 67 tested individuals had a fourfold or higher viral load in saliva relative to that in plasma (27).

Several endogenous oral components are proposed to contribute to inactivating HIV, either alone or in combination (28). Among them, the secretory leukocyte protease inhibitor has been extensively studied. The secretory leukocyte protease inhibitor was first described by Thompson and Ohlsson (30) as a potent inhibitor of leukocyte elastase. Since then, studies from many independent laboratories have confirmed its anti-HIV-1 activity in in vitro assays (17, 19, 28). Recently, a novel mechanism of anti-HIV activity has been proposed; this mechanism involves the unique hypotonicity of saliva (2, 3). The toxicity of saliva is approximately 14% of that of blood and other mucosal secretions. Peripheral blood mononuclear cells were rapidly disrupted in saliva in an in vitro study. The hypothesis is that cell-associated HIV is released due to saliva disruption, and cell-free HIV is mostly neutralized by a specific antibody or inhibited by other macromolecular inhibitors in saliva.

Despite the great effort that has been made to search for oral inhibitors of HIV, little information is available on the interaction of HIV and bacteria in the oral cavity, an environment harboring over 30 genera representing more than 500 species of bacteria (21). We speculated that oral bacteria may contain anti-HIV activity and create a microenvironment unfavorable for HIV infection. In this study, members of a group of oral bacteria were examined for their abilities to inhibit HIV-1. We demonstrated that Porphyromonas gingivalis, an organism frequently detected in the mouths of adult periodontitis patients, produced an antiviral molecule(s) which was capable of inhibiting HIV-1 infection. In an attempt to identify the inhibitor activity, HGP44, the binding domain of Arg-gingipain-1 of P. gingivalis, was found to inhibit HIV-1 infection by preventing interaction between envelope glycoprotein 120 (gp120) and cell receptors.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Actinobacillus actinomycetemcomitans, Prevotella intermedia, Fusobacterium nucleatum, and Porphyromonas gingivalis were grown from frozen stocks in Trypticase soy broth (TSB; BBL, Cockeysville, MD) or on TSB blood agar plates supplemented with yeast extract (1 mg/ml; Difco, Detroit, MI), hemin (5 μg/ml; Sigma, Dallas, TX), and menadione (1 μg/ml; Sigma, Dallas, TX) at 37°C in an anaerobic chamber (85% N2, 10% H2, 5% CO2). Streptococcus gordonii, Streptococcus cristatus, and Actinomyces viscosus strains were grown in Trypticase peptone broth (BBL, Cockeysville, MD) supplemented with 0.5% glucose at 37°C under aerobic conditions. Escherichia coli strains were grown in L broth at 37°C. Antibiotics were purchased from Sigma, Dallas, TX, and used when appropriate at the following concentrations: gentamicin, 100 μg/ml; erythromycin, 20 μg/ml; ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 20 μg/ml.

Cell fusion assay. The fusion assays were performed by transfecting monkey kidney cells (COS) with the expression vector pSRHS that contains the HIV-1 NL4-3 envelope gene. The original HXB envelope in pSRHS was replaced with the NL4-3 envelope. Electroporation was performed to express the HIV-1 envelope in COS cells. COS cells (10⁶) in culture medium were incubated with 2 μg of the envelope expression vector on ice for 10 min. The electroporation was performed using a gene pulser (Bio-Rad, Hercules, CA) with capacitance set at 950 μF and a voltage of 150 V. The transfected COS cells were cultured for 1 day before being mixed with the fusion partner TZM cells (24). The TZM cells (7 × 10⁴) were incubated with the envelope-transfected cells (10⁶) in 96-well, flat-bottomed plates (Costar) in 100 μl culture medium. Samples to be tested at various concentrations in 10 μl of culture medium were incubated with the cell mixtures at 37°C for 24 h. A Promega luciferase assay kit was used to quantify luciferase activity in the fused cells using a BioTek luminometer. The concentration that reduced the control HIV-1 envelope-mediated fusion by 50% is defined as the 50% effective concentration.

HIV-1 infectivity assay. A previously described HIV-1 infectivity assay was used in the experiments (13). A 96-well microtiter plate was set up to test the HIV-1 NL4-3 replication assay. HIV-1 NL4-3 at a multiplicity of infection of 0.01 was used to infect MT4 cells. Culture supernatants were collected on day 4 postinfection for a p24 assay using an enzyme-linked immunosorbent assay (ELISA) kit from PerkinElmer Life Sciences (Wellesley, MA).

Initial purification of inhibitory proteins. P. gingivalis 33277 was cultured to late log phase, and cells were collected by centrifugation and resuspended in...
phosphate-buffered saline (PBS). A bacterial surface extract was prepared by sonication with a sonic dismembrator (Fisher Scientific output control 8; three times for 30 s), and cell debris was removed by centrifugation (13,000 × g for 10 min), followed by filtration (0.2-μm pore size). The bacterial surface extract was precipitated by using ammonium sulfate (37% saturation). The precipitated protein mixture was dialyzed against PBS (pH 7.5), filtered (0.22 μm), and applied to a HiTrap DEAE column (1 × 5 cm) (Amersham) equilibrated with the same buffer. The column was washed with the buffer until the optical density at 280 nm was less than 0.03. Proteins were then eluted with a linear concentration gradient of NaCl from 0 to 500 mM in PBS at a flow rate of 1 ml/min using a Biologic LP chromatography system (Bio-Rad). The proteins in the eluent were detected with a UV monitor at 280 nm and collected in 3-ml fractions. The fractions were dialyzed against PBS and assayed for anti-HIV-1 entry activity.

Affinity chromatography of DEAE-U fraction on Sepharose 4B coupled to gp120. A bacculovirus-expressed HIV-1 gp120 (34) was coupled to CNBr-activated Sepharose 4B according to the procedure provided by the manufacturer (Pharmacia Biotech). The active fraction from DEAE fractionation was incubated with gp120-coupled Sepharose 4B for 30 min in the presence of protease inhibitors (Protease Arrest; Geno Technology, Inc). The column was washed with PBS until the optical density at 280 nm of the eluate was less than 0.02. The binding fraction was then eluted with 4 M MgCl2. The eluate was dialyzed against PBS (pH 7.5) and concentrated.

Labeling of gp120 with 1125I and binding of 125I-gp120 to proteins on nitrocellulose. HIV-1 gp120 was produced using a baculovirus expression system as previously described (34). The purified gp120 was labeled with 125I in the presence of Iodo-Beads purchased from Pierce Chemical Company (Rockford, Ill.) following the manufacturer’s protocol. For the binding assay, proteins were resolved by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS-12% PAGE) and transferred to a nitrocellulose membrane. After blocking with 2% bovine serum albumin (BSA), the membrane was incubated with 125I-labeled gp120 (10 106 cpm) at room temperature for 2 h. The membrane was washed three times with PBS containing 2% BSA and 0.5% Tween 20. The membrane was then exposed to Kodak X-OMAT film (Sigma) for 2 days.

HGP44 cloning and expression. A DNA fragment of the mg4 encoding HGP44 was amplified by PCR with primers 5'-AGGAAATTCTCCCGGACTTCTT (EcoRI site is underlined) and 5'-AGACTCGAACGTCGACGTGAA (SalI site is underlined), which produced a 1,302-bp PCR product. The PCR products were then cloned into pCRII-TOPO (Invitrogen, Carlsbad, CA). Recombinant HGP44 (rHGP44) was expressed in the expression system (Novagen, Madison, WI). The HGP44 DNA fragment was inserted into expression vector pET-30b downstream of a histidine tag. The recombinant HGP44 was expressed in E. coli BL21(DE3) cells carrying the pET-30b-HGP44 plasmid in the presence of IPTG (isopropyl-β-thiogalactopyranoside) and kanamycin. His-tagged rHGP44 was purified with Ni2+ charged His-Bind resin (Novagen, Madison, WI). The His tag on the recombinant protein was cleaved with enterokinase and removed by His-Bind resin. Enterokinase was then removed by using EKapture agarose.

ELISA. ELISA plates (Costar) were coated with testing proteins (10 μg/ml). Plates were sealed and incubated at 4°C overnight and then washed five times with PBS containing 0.1% Tween 20, pH 7.4 (TPBS). Plates were blocked with TPBS containing 3% BSA for 1 h at 37°C. A series of dilutions of M41 or gp120 were then added and incubated at room temperature for 1 h. M41 is a recombinant protein derived from the ectodomain of HIV-1 gp120 (6). The plates were washed with PBS and incubated with primary antibody anti-rabbit anti-MBP or sheep anti-gp120 for 1 h. After washing away the unbound antibodies, horse-radish peroxidase-conjugated antibodies were added to detect the binding of primary antibodies. All samples were assayed in triplicate and at least two times with different plates.

Statistical analysis. Statistical analysis of anti-HIV activities and interaction of HGP44 and gp120 was performed by using analysis of covariance, analysis of variance, and Bonferroni tests in SAS (version 9.1; SAS Institute, Cary, NC). P values of <0.05 were considered statistically significant.

RESULTS

Antiviral activity of P. gingivalis. In order to examine the abilities of oral bacteria to inhibit HIV-1 infection, a cell-cell fusion assay was used to evaluate the effects of the bacterial extracts on viral envelope-mediated HIV-1 entry. We screened the effect of a group of oral bacteria strains, including Streptococcus gordonii G9B, S. cristatus CC5A, Actinomyces viscosus NC-3, Actinobacillus actinomycetemcomitans Y4, Prevotella intermedia 27, Fusobacterium nucleatum 25858, and Porphyromonas gingivalis 33277, on HIV-1 envelope-mediated cell-cell fusion. The bacteria were grown in appropriate media to late log phase. Bacterial surface extracts were isolated by sonication and centrifugation. The extracts were then assayed for their effects on HIV-1 envelope-mediated cell-cell fusion. Among the tested oral bacteria, only the P. gingivalis extract exhibited potent antifusion activity (P = 0.001) (Fig. 1), suggesting that the extract from P. gingivalis interfered with the interaction of the viral envelope glycoproteins and the cellular receptors. The cytotoxicity of the bacterial extract was analyzed using a trypan blue exclusion assay. In a 1-day cytotoxicity assay, bacterial extract from P. gingivalis decreased the viability of COS and TZM cells by 50% at 45 μg/ml. The bacterial extract could inhibit HIV-1 envelope-mediated fusion without cytotoxicity. In contrast, the extracts of the other tested bacteria, including Streptococcus gordonii G9B and Actinobacillus actinomycetemcomitans Y4, did not affect HIV-1 envelope-mediated membrane fusion. To determine the nature of the inhibitory molecule, P. gingivalis extract was subjected to heat treatment by boiling for 20 min. As shown in Fig. 1, heat treatment abolished the antifusion activity of P. gingivalis extract, suggesting that proteins were involved. To test whether P. gingivalis extracts can indeed inhibit HIV-1 infection, we examined the bacterial extracts in an HIV-1 replication assay. The extracts of P. gingivalis exhibited potent anti-HIV activity against NL4-3 virus (Fig. 2). The concentration that inhibited the virus replication by 50% was 10 μg/ml of the crude extract. The bacterial extract inhibited the viability of MT4 cells by 50% at 30 μg/ml in a 4-day assay. This concentration is approximately threefold higher than that required to inhibit HIV-1 replication. The relatively low selectivity of the bacterial extract might be due to the presence of bacterial components that are toxic to P. gingivalis gingipains and anti-HIV-1 agent. 3071

FIG. 1. Antifusion activity of bacterial extracts. Extracts isolated from A. actinomycetemcomitans Y4, S. gordonii G9B, and P. gingivalis 33277 were tested for their abilities to inhibit HIV entry by cell fusion assays. P. gingivalis 33277 extract (50 μg/ml) inhibited >98% of cell fusion. In contrast, extracts of A. actinomycetemcomitans Y4 and S. gordonii G9B had no effect on cell fusion. Heat treatment completely abolished the inhibitory activity of P. gingivalis 33277 extract. Each data point in the figure represents the mean ± standard deviation from at least three different experiments.
to the cells. IC9564 is used in the experiment as a positive control. IC9564 is a small-molecule HIV-1 entry inhibitor that can block HIV-1 entry by targeting HIV-1 envelope glycoproteins (13).

To determine whether the bacterial component(s) was expressed on the surface of the bacterium, *P. gingivalis* 33277 was grown in TSB for 24 h, collected by centrifugation, and resuspended in PBS. The bacterial cells were added in the fusion assays in the presence of tetracycline (1 μg/ml) to prevent the replication of the organism. The concentration of *P. gingivalis* required to inhibit 50% of the cell-cell fusion was 2.2 × 10^6/ml (data not shown). These findings suggested that a component(s) on the surface of the organism or secreted by the organism was involved in the anti-HIV-1 activity.

**Purification and identification of *P. gingivalis* inhibitory proteins.** To identify the HIV inhibitory protein, the crude surface extract of *P. gingivalis* was first fractionated by using ammonium sulfate [(NH₄)₂SO₄] precipitation. Each fraction was precipitated by a series of saturated (NH₄)₂SO₄ and tested for its inhibitory activity with a fusion assay. The effect of the fraction on HIV entry and infection was found in the fraction precipitated with 37% saturated (NH₄)₂SO₄. The active fraction was then subjected to DEAE ion exchange chromatography for further purification. The anti-HIV-1 activity was found in the DEAE unbound fraction.

Since gp120 and gp41 are the two viral proteins essential for HIV-1 entry, we hypothesized that the inhibitory proteins of *P. gingivalis* interacted with one or both of the proteins to block HIV-1 entry. To test this hypothesis, a simple ELISA was used to determine the interaction between the bacterial proteins and the viral envelope proteins gp120 and gp41. The ELISA plates coated with a recombinant gp41 protein (M41) (6) or a baculovirus-expressed gp120 (34) were incubated with various concentrations of *P. gingivalis*, active fractions of ammonium sulfate precipitation. The results of ELISA demonstrated that proteins in *P. gingivalis* surface extracts bound to gp120 (data not shown). To isolate the gp120 binding protein from *P. gingivalis*, gp120 was coupled to Sepharose 4B for affinity purification. A protein with molecular mass of 45 kDa eluted from the affinity column was revealed by SDS-PAGE (Fig. 3A).

To verify that this 45-kDa protein indeed binds to gp120, the purified proteins were transferred to a nitrocellulose membrane for a binding assay using ^125^I-labeled gp120. As shown in Fig. 3B, only a 45-kDa protein interacted with ^125^I-labeled gp120, suggesting that this *P. gingivalis* protein could indeed interact with gp120.

To determine the identity of the 45-kDa protein, the protein eluted from the affinity column was subjected to Edman sequencing to obtain the N-terminal amino acid sequence of the protein. The first nine amino acids were determined as GQA, which is a 100% match with the adhesion domain HGP44 of *P. gingivalis* arginine-specific cysteine proteinase (arginipain, RgpA). HGP44 is a major adhesion domain derived from gingipains due to autoproteolysis. Gingipains are processed by their own proteolytic domains. After cleavage, the individual domains are noncovalently associated as complexes displayed on the surfaces of the organisms (22).

**Adhesive domain of *P. gingivalis* gingipain inhibited HIV-1 replication.** Gingipains are a unique family of enzymes in *P. gingivalis*. Two major gingipains are Arg-gingipain (RgpA) and Lys-gingipain (Kgp) (7). Both RgpA and Kgp constitute a specific catalytic domain and four C-terminal domains which have >98% amino acid identity between the two gingipains (Fig. 4). These C-terminal domains are also known as adhesion domains which bind to hemoglobin, the oral bacterium *Prevotella intermedia*, and epithelial cells (7, 15). To further examine whether the adhesion domain HGP44 contributed to the anti-HIV activity, a recombinant HGP44 was generated by expressing the HGP44 domain as a His-tagged protein in *E. coli* BLC1. After removal of the His tag, purified HGP44 was tested for anti-HIV and antientry activities. The rHGP44 inhibited HIV-1 NL4-3 at a 50% effective concentration of approximately 2.5 μM (P = 0.002) (Fig. 5). In the light of the potent activity of anti-HIV-1 in *P. gingivalis* extracts, this anti-HIV activity of rHGP was not as potent as we expected. This might be due to differences between recombinant protein and
the native proteins in the bacterial extract. As a control, the recombinant FimA, a major surface adhesive protein of *P. gingivalis*, was also tested for its effect on HIV infectivity. FimA did not inhibit HIV-1 replication. Instead, FimA slightly increased HIV infectivity. The rHGP44 binding activity to gp120 was confirmed by using ELISA. The affinities of rHGP44, rFimA, and bovine serum albumin to gp120 were tested. As shown in Fig. 6, the recombinant HGP44 bound well to gp120 (P < 0.0001). There was little statistically significant interaction between other proteins and gp120. We also found that HGP44 did not bind to M41 (P < 0.0001).

**DISCUSSION**

A variety of natural products isolated from microorganisms have been described as anti-HIV agents. Chokekijchai et al. (9) screened 9,000 extracts of bacterial samples from various sources for anti-HIV activity. A crude extract of strain SKH-2344, a *Streptomyces* strain isolated from soil, showed high anti-HIV activity. Further investigation led to the identification of a 21-mer oligopeptide, NP-06, that appears to block HIV-1 infection at the stage of virus-cell fusion. Several peptides structurally similar to NP-06 were isolated from *Streptomyces* by independent laboratories and showed strong inhibitions of virus-cell fusion (11, 18). Anti-HIV agents from other bacteria, such as actinohivin from an actinomycete, were also found (8). In addition, cyanovirin-N isolated from the cyanobacterium *Nostoc ellipso sporum* was shown to have potent anti-HIV activity (4). This 11-kDa protein exerts broad antiviral activity and is currently under clinical trial as a topical anti-HIV drug candidate. Similar to most anti-HIV agents isolated from bacteria, *P. gingivalis* HGP44 inhibits HIV-1 at the very early stage of the HIV replication cycle, which includes sequential interactions between HIV-1 envelope glycoproteins and host cell receptors.

*P. gingivalis* is closely associated with adult periodontitis. Previous studies have consistently shown that *P. gingivalis* was significantly more prevalent in both supra- and subgingival plaque samples from periodontitis subjects compared to those from healthy individuals (10, 31). 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 noninfected control subjects with similar periodontal statuses (5, 20, 26, 33). On the contrary, recent results from Patel et al. (23) showed a significant prevalence of *Porphyromonas gingivalis* and *Treponema denticola* among HIV-negative patients compared to that among HIV-positive patients. Thus, it is possible that *P. gingivalis* and HIV infections might coexist in the oral cavity, especially in HIV-positive periodontitis patients.

The gingipains (RgpA and Kgp) are cell surface Arg- and Lys-specific proteases of *P. gingivalis*. RgpA and Kgp, encoded by *rgpA* and *kgp* genes, respectively, are polyproteins comprising a proteolytic domain and four C-terminal adhesin domains (Fig. 4) (1). These domains formed a complex on the surface of the bacteria after autoproteolysis (22). Although the gingipains are also purified from the culture supernatant of *P. gingivalis* (14), the cell-associated gingipains comprise the majority (~80%) of Rgp and Kgp (29). RgpA and Kgp are con-
sidered critical virulence factors in *P. gingivalis* pathogenicity. The proteolytic domains appear to be involved in periodontal tissue destruction and host defense inactivation (12). The adhesin domains are thought to be responsible for *P. gingivalis* colonization in oral epithelial cells (7) and other oral bacteria, such as *Prevotella intermedia* (15). The results of this study indicated that HGP44 of *P. gingivalis* could bind to gp120 and inhibited HIV-1 entry. The specific binding of *P. gingivalis* HGP44 to gp120 glycoprotein of HIV appears to be, at least in part, the mechanism by which gingipains inhibit HIV entry, a first step of HIV infection. Kitamura et al. (16) reported that gingipains, including high-molecular-mass arginine-specific gingipain, arginine-specific gingipain B, and lysine-specific gingipain, can actively cleave CD4 and CD8 on human T cells. Thus, it is possible that both proteolytic and binding domains of gingipains might inhibit HIV-1 infection. This may be one of the reasons that rHGP44 alone is not as potent as expected. Based on these findings, we propose a model for *P. gingivalis* and HIV-1 co-infection. The RgpA proteolytic domain could cleave HIV-1 receptor CD4. HGP44 of RgpA can also bind to HIV-1 gp120 before gp120 interacts with CD4. The protease activity and gp120 binding activity could synergize and render RgpA a potent anti-HIV molecule.

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