Anti-*Porphyromonas gingivalis* and Anti-Inflammatory Activities of A-Type Cranberry Proanthocyanidins[▽]

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A-type cranberry proanthocyanidins (AC-PACs) have recently been reported to be beneficial for human health, especially urinary tract health. The effect of these proanthocyanidins on periodontitis, a destructive disease of tooth-supporting tissues, needs to be investigated. The purpose of this study was to investigate the effects of AC-PACs on various virulence determinants of *Porphyromonas gingivalis* as well as on the inflammatory response of oral epithelial cells stimulated by this periodontopathogen. We examined the effects of AC-PACs on *P. gingivalis* growth and biofilm formation, adherence to human oral epithelial cells and protein-coated surfaces, collagenase activity, and invasiveness. We also tested the ability of AC-PACs to modulate the *P. gingivalis*-induced inflammatory response by human oral epithelial cells. Our results showed that while AC-PACs neutralized all the virulence properties of *P. gingivalis* in a dose-dependent fashion, they did not interfere with growth. They also inhibited the secretion of interleukin-8 (IL-8) and chemokine (C-C motif) ligand 5 (CCL5) but did not affect the secretion of IL-6 by epithelial cells stimulated with *P. gingivalis*. This anti-inflammatory effect was associated with reduced activation of the nuclear factor-κB (NF-κB) p65 pathway. AC-PACs may be potentially valuable bioactive molecules for the development of new strategies to treat and prevent *P. gingivalis*-associated periodontal diseases.

Periodontitis is a multifactorial polymicrobial infection characterized by a destructive inflammatory process resulting in the loss of tooth-supporting tissues. Approximately 5 to 15% of the population is affected by severe forms of the disease which, if left untreated, may result in tooth loss and systemic complications (8). The Gram-negative bacterium *Porphyromonas gingivalis* is a key etiologic agent of periodontitis, more particularly the chronic form (41). *P. gingivalis* is able to adhere to cellular and acellular surfaces (32) and form a biofilm (33), both of which contribute to its establishment in and colonization of the oral cavity. *P. gingivalis* also produces soluble and cell-bound proteases (24) that can degrade various tissue and plasma proteins and that contribute to the invasion of periodontal tissues (1).

The gingival epithelium has a stratified squamous structure that is an interface between the external environment, with its complex bacterial ecosystem, and the underlying periodontal tissue. *P. gingivalis* has developed different strategies to colonize and perturb the structural and functional integrity of the gingival epithelium (2). It is capable of inducing a strong proinflammatory cytokine response in gingival epithelial cells *in vitro*, which has been correlated with the adhesive/invasive potential of *P. gingivalis* (39, 40).

The cranberry (*Vaccinium macrocarpon* Ait.) is a native North American fruit that has recently received considerable attention in health research, particularly in the field of infectious diseases. While proanthocyanidins are found in all berry fruits, those isolated from cranberry possess unusual structures with A-type linkages, with a second ether linkage between an A-ring of the lower unit and the C-2 ring of the upper unit (O7 → C2) (14). These A-type cranberry proanthocyanidins (AC-PACs) are composed mainly of epicatechin units, with at least one A-type linkage (13). A-type linkages can occur at the terminal unit or between the extension units (18). AC-PAC oligomers and polymers with up to 12 degrees of polymerization and as many as four A-type linkages have been detected using matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) (37). While AC-PACs inhibit the adherence properties of uropathogenic *Escherichia coli* (23), which may explain the beneficial effect of cranberry for preventing urinary tract infections in children (12) and aging patients (36), the B-linked proanthocyanidins isolated from apple juice, green tea, and dark chocolate have no antiadhesion activity (23). A number of studies have suggested that cranberry polyphenols may promote oral health by inhibiting dental biofilm formation (31, 42, 44), acid production by *Streptococcus mutans* (11), periodontopathogen-derived proteolytic enzymes (6), and host inflammatory responses (4), but they suffer from the fact that poorly characterized fractions have been used.

In the present study, we hypothesized that AC-PACs may have beneficial effects for *P. gingivalis*-associated periodontal diseases. We thus investigated the effects of AC-PACs on *P. gingivalis* growth and biofilm formation, adherence to human oral epithelial cells and protein-coated surfaces, collagenase activity, and invasiveness. We also investigated the anti-inflammatory effects of AC-PACs in oral epithelial cells stimulated by *P. gingivalis*.

MATERIALS AND METHODS

A-type cranberry proanthocyanidins. Cranberry proanthocyanidins were isolated from cranberry fruit (*Vaccinium macrocarpon* Ait.) using solid-phase chromatography according to a well-established method for proanthocyanidin isola-

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tion (23). Briefly, cranberry fruit was homogenized with 70% aqueous acetone and filtered, and the pulp was discarded. The collected extract was concentrated under reduced pressure to remove acetone. The cranberry extract was suspended in water, applied to a preconditioned C_{18} solid-phase chromatography column, and washed with water to remove sugars, followed by acidified aqueous methanol to remove acids. The fats and waxes retained on the C_{18} sorbent were discarded. The polyphenolic fraction containing anthocyanins, flavonol glycosides, and proanthocyanidins (confirmed using reverse-phase high-pressure liquid chromatography [HPLC] with diode array detection) was eluted with 100% methanol and dried under reduced pressure. This fraction was suspended in 50% ethanol (EtOH) and applied to a preconditioned Sephadex LH-20 column which was washed with 50% EtOH to remove low-molecular-weight anthocyanins and flavonol glycosides. Proanthocyanidins adsorbed to the LH-20 were eluted from the column with 70% aqueous acetone and monitored using diode array detection at 280 nm. The absence of absorption at 360 nm and 450 nm confirmed that anthocyanins and flavonol glycosides were removed. Acetone was removed under reduced pressure and the resulting purified proanthocyanidin extract freeze-dried. ^{13}C nuclear magnetic resonance (NMR), electrospray mass spectrometry, matrix-assisted laser desorption ionization–time-of-flight mass spectrometry, and acid-catalyzed degradation with phloroglucinol have all been utilized to confirm the presence of A-type linkages and concentration of proanthocyanidins present in the extract (13, 14, 23).

Bacterium. *P. gingivalis* ATCC 33277 was grown in Todd-Hewitt broth (THB) (BBL Microbiology Systems, Cockeysville, MD) supplemented with 0.001% hemin and 0.0001% vitamin K (THB-HK). Cultures were incubated at 37°C under anaerobic conditions (N_2-H_2-CO_2, 80:10:10).

Growth and biofilm formation. A 24-h culture of *P. gingivalis* in THB-HK was diluted in fresh broth medium to obtain an optical density at 655 nm (OD_{655}) of 0.2. Equal volumes (100 μl) of *P. gingivalis* and AC-PACs (0, 50, 100, and 200 μg/ml) in THB-HK were mixed in the wells of 96-well plates (Sarstedt, Newton, NC). Control wells with no *P. gingivalis* were also prepared. After a 24-h incubation at 37°C under anaerobic conditions, bacterial growth was recorded by measuring the OD_{655} using a microplate reader. The spent medium and free-floating bacteria were then removed by aspiration, and the wells were washed three times with distilled water. The *P. gingivalis* biofilms were stained with 100 μl of 0.4% crystal violet for 15 min. The wells were washed four times with distilled water to remove the unbound crystal violet dye and were dried for 2 h at 37°C. After addition of 100 μl of 95% ethanol to each well, the plate was shaken for 10 min to release the stain from the biofilms. The absorbance at 550 nm (A_{550}) was measured to quantify biofilm formation.

Adherence to human oral epithelial cells and extracellular matrix proteins. *P. gingivalis* cells were radiolabeled by incubating a mid-log-phase culture (OD_{655} of 0.5) with a mixture of ^{14}C-labeled amino acids (Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, United Kingdom) at a final concentration of 20 μCi/ml at 37°C for 16 h in an anaerobic chamber. Cells were harvested by centrifugation at 10,000 × g for 10 min, washed three times in 10 mM phosphate-buffered saline (PBS) (pH 7.2), and suspended to an OD_{655} of 0.8 in PBS prereduced by overnight incubation in the anaerobic chamber. The immortalized human oral epithelial cell line GMSM-K (developed by Valerie Murrah, Department of Diagnostic Sciences and General Dentistry, University of North Carolina at Chapel Hill), which has an epithelial phenotype (17), was used to investigate the effect of AC-PACs on the adherence of *P. gingivalis*. GMSM-K cells were cultured onto wells of 96-well plates (Sarstedt) in Dulbecco's modified Eagle's medium (DMEM) to form a confluent monolayer as reported elsewhere (30). The wells were washed three times with PBS prior to performance of the adherence assay. Other wells of 96-well flat-bottomed microtiter plates were filled with 100 μl of Matrigel (Sigma-Aldrich, St. Louis, MO) diluted 1/10 in ice-cold PBS. The Matrigel was allowed to settle at room temperature for 2 h and was then washed three times with distilled water prior to the adherence assay. Matrigel is composed of several extracellular matrix proteins, including laminin, type IV collagen, heparan sulfate proteoglycans, and entactin. Mixtures containing 20 μl of ^{14}C-labeled *P. gingivalis* (OD_{655} of 0.8) and 30 μl of AC-PACs in PBS were prepared to obtain a final AC-PAC concentration of 0, 25, 50, or 100 μg/ml. The mixtures were placed in wells coated with epithelial cells or extracellular matrix proteins. After a 30-min incubation at 37°C, the wells were washed three times with PBS to remove unbound bacteria. Adhered radiolabeled bacteria were detached by adding 100 μl of 0.5 M NaOH and incubating the plates at room temperature for 15 min on an orbital shaker. The bacteria were suspended in EcoLite scintillation liquid (ICN, Costa Mesa, CA), and the radioactivity was counted using a multipurpose scintillation counter (Beckman Coulter, Fullerton, CA).

Degradation of type I collagen. A cell-free culture supernatant of *P. gingivalis* was obtained from a 36-h culture. Aliquots of supernatant (7.5 μl) were mixed with 127.5 μl of TCNB (50 mM Tris HCl, 10 mM $CaCl_2$, 150 mM NaCl, and 0.05% Brij 35, pH 7.5) buffer containing increasing concentrations of AC-PACs (final concentrations of 0, 25, 50, and 100 μg/ml) and 15 μl of 1-mg/ml fluorogenic substrate (fluorescein-conjugated DQ type I collagen; Molecular Probes, Eugene, OR). The assay mixtures were incubated in the dark at room temperature, and the fluorescence was measured every hour for 4 h using a fluorometer with the excitation and emission wavelengths set at 490 nm and 520 nm, respectively.

Invasion of a reconstituted basement membrane model. One hundred microliters of Matrigel diluted 1/2 in ice-cold PBS was placed on 8-μm-pore-size filters in Transwell cell culture chamber inserts (Costar, Cambridge, MA), while 400 μl of PBS was added to the lower chamber. The Matrigel was allowed to settle at 4°C for 30 min and then to form a gel at 37°C for 2 h in an anaerobic chamber (N_2-H_2-CO_2, 80:10:10) prior to performing the invasion assay. Fifty microliters of ^{14}C-labeled *P. gingivalis* cells (OD_{655} of 0.16) was pretreated with AC-PACs at a final concentration of 0, 25, 50, or 100 μg/ml for 30 min and then placed on top of the Matrigel in the double-chamber system, which was incubated in the anaerobic chamber at 37°C for 48 h. The migration of *P. gingivalis* cells through the reconstituted basement membrane model was monitored by measuring the radioactivity in the buffer recovered from the lower chamber after the incubation period.

Inflammatory response of an oral epithelial cell model. The oral epithelial cells (GMSM-K) were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics, harvested by gentle trypsinization (0.05% trypsin-EDTA; Gibco-BRL, Grand Island, NY), washed once in DMEM-FBS, and suspended at a density of 4×10^5 cells per ml in DMEM with 1% heat-inactivated FBS (30). Cells were seeded in a 12-well plate (4×10^5 cells/well in 1 ml) and cultured overnight at 37°C in a 5% CO_2 atmosphere to allow cell adhesion prior to the stimulation with *P. gingivalis*. The epithelial cells were pretreated with increasing concentrations of AC-PACs (0, 25, 50, and 100 μg/ml) and were incubated at 37°C in 5% CO_2 for 2 h before the stimulation with *P. gingivalis* cells at a multiplicity of infection (MOI) of 125. This MOI was selected on the basis of preliminary experiments showing that it induced cytokine secretion by epithelial cells while not affecting their viability, as determined by an MTT (3-[4,5-diethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (data not shown). After a 24-h incubation at 37°C in 5% CO_2, cell-free supernatants were collected and stored at −20°C until used. Commercial enzyme-linked immunosorbent assay (ELISA) kits (R & D Systems, Minneapolis, MN) were used to quantify interleukin-6 (IL-6), interleukin-8 (IL-8), and chemokine (C-C motif) ligand 5 (CCL5) concentrations in the free-cell supernatants according to the manufacturer's protocols. The absorbance at 450 nm was read using a microplate reader with the wavelength correction set at 550 nm. The rated sensitivities of the commercial ELISA kits were 9.3 pg/ml for IL-6, 31.2 pg/ml for IL-8, and 15.6 pg/ml for CCL5.

To understand the mechanism of action of AC-PACs, their effect on nuclear factor-κB (NF-κB) p65 activation was investigated. Epithelial cells prepared as described above were incubated in the absence or presence of AC-PACs (50 μg/ml) for 1 h prior to being stimulated with *P. gingivalis* cells at an MOI of 125 for 30 min. Whole-cell extracts were then prepared using nuclear extract kits (Active Motif, Carlsbad, CA) according to the manufacturer's protocol and were adjusted to a protein concentration of 1 mg/ml. NF-κB p65 binding to DNA in the cell extracts was determined using TransAm NF-κB p65 kits (Active Motif) according to the manufacturer's protocol.

Statistical analysis. Data are expressed as the means ± standard deviations (SD) from at least three assays. The statistical analysis was conducted using the Student t test with Bonferroni corrections. A P value of <0.05 was considered statistically significant.

RESULTS

The characterization of the AC-PAC fraction was made by ^{13}C NMR. As shown in Fig. 1, the proanthocyanidin molecules consist of epicatechin units with degrees of polymerization (DP) mainly of 4 and 5 containing at least one A-type linkage, as previously reported (13) (Fig. 1).

To investigate the effect of AC-PACs on various pathogenic properties of *P. gingivalis*, up to 100 μg/ml of AC-PACs was used, since this concentration has been reported to be nontoxic for human cells (29). Biofilm formation by *P. gingivalis* was significantly decreased by 50 and 100 μg/ml AC-PACs. More
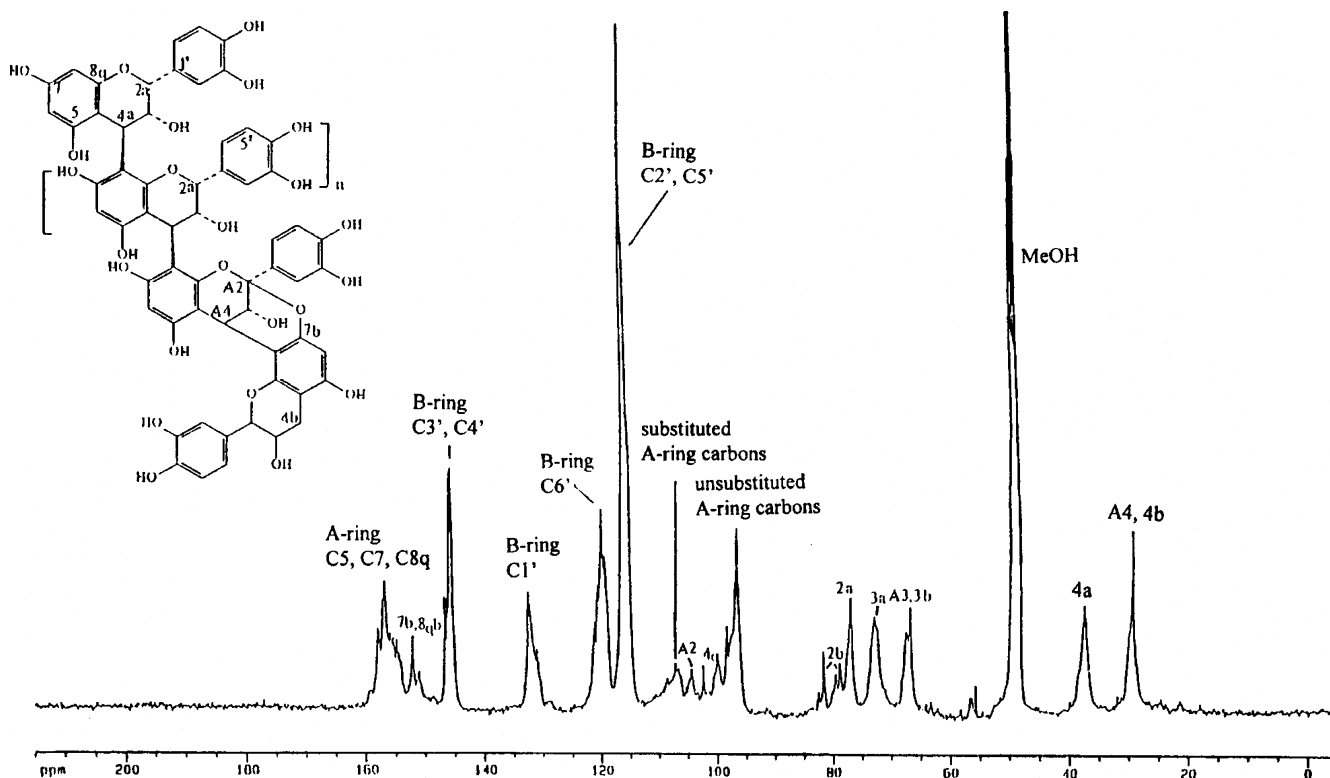
FIG. 1. ^{13}C nuclear magnetic resonance (NMR) spectrum of cranberry proanthocyanidins, showing the presence of A-type linkages.

specifically, AC-PACs inhibited biofilm formation by 45% ± 6% and 60% ± 4% at concentrations of 50 and 100 μg/ml, respectively (Fig. 2). AC-PACs at all the concentrations tested did not significantly affect the growth of *P. gingivalis*.

The effect of AC-PACs on the adherence properties of *P. gingivalis* was investigated using microplate wells covered with confluent oral epithelial cells or coated with extracellular matrix proteins (Matrigel). The relative adherence of ^{14}C-labeled
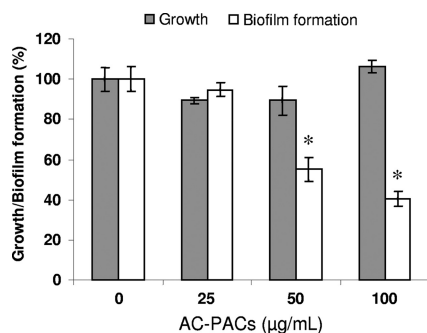
FIG. 2. Effect of AC-PACs on the growth of and biofilm formation by *P. gingivalis*. AC-PACs (0, 25, 50, and 100 μg/ml) were added to the growth medium, and the cultures were incubated under anaerobic conditions at 37°C for 24 h. Bacterial growth was assessed by measuring the OD_{655} using a microplate reader. Biofilm formation was assessed by staining with 0.4% crystal violet and measuring the A_{550}. Assays were run in quadruplicate, and the means ± SD from three independent assays were calculated. A value of 100% was assigned to growth and biofilm formation in the absence of AC-PACs. *, significantly lower than the value for the control (no AC-PACs) ($P < 0.05$).

P. gingivalis to oral epithelial cells was not significantly decreased at the lowest concentration of AC-PACs tested (25 μg/ml). However, 50 and 100 μg/ml of AC-PACs inhibited *P. gingivalis* adherence to epithelial cells by 37.5% ± 7.0% and 54.1% ± 8.2%, respectively. AC-PACs also inhibited the adherence of *P. gingivalis* to Matrigel-coated polystyrene surfaces, with 25, 50, and 100 μg/ml inhibiting adherence by 48.0% ± 5.8%, 64.1% ± 7.7%, and 78.9% ± 2.2%, respectively (Fig. 3).

The ability of AC-PACs to inhibit type I collagen degradation by extracellular proteinases produced by *P. gingivalis* was also tested. Significant inhibition was observed at all the concentrations tested, with 25, 50, and 100 μg/ml of AC-PACs inhibiting type I collagen degradation by a *P. gingivalis* culture supernatant by 50.2% ± 4.4%, 73.7% ± 6.8%, and 88.7% ± 8.5%, respectively (Fig. 4A). This inhibitory effect of AC-PACs on type I collagen degradation was time dependent (Fig. 4B).

The effect of AC-PACs on the invasiveness of *P. gingivalis* was investigated using a reconstituted basement membrane model (Matrigel) in a double-chamber system. The migration of ^{14}C-labeled *P. gingivalis* cells through the Matrigel was reduced by 23.9% ± 3.6% and 27.5% ± 3.0% in the presence of 50 and 100 μg/ml AC-PACs, respectively (Fig. 5).

The ability of AC-PACs to modulate the *P. gingivalis*-induced inflammatory response of oral epithelial cells was then examined. Epithelial cells were treated with AC-PACs for 2 h prior to being stimulated with cells of *P. gingivalis* to investigate their effects on IL-6, IL-8, and CCL5 secretion. At an MOI of 125, *P. gingivalis* significantly increased the secretion of IL-6, IL-8, and CCL5 (Fig. 6A), while having no effect on epithelial
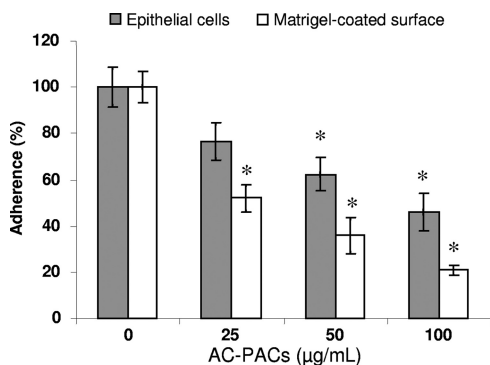
FIG. 3. Effect of AC-PACs on the adherence of *P. gingivalis* to human oral epithelial cells and a Matrigel-coated polystyrene surface. ^{14}C-labeled *P. gingivalis* cells in the presence of AC-PACs (0, 25, 50, and 100 μg/ml) were added to wells coated with epithelial cells or Matrigel. After 30 min of incubation at 37°C, the wells were extensively washed and the quantity of adhered ^{14}C-labeled bacteria was determined by gamma counting using a multipurpose scintillation counter. Assays were run in triplicate, and the means ± SD from three independent assays were calculated. A value of 100% was assigned to the amount of ^{14}C bound in the absence of AC-PACs. *, significantly lower than the value for the untreated control ($P < 0.05$).

cell viability. AC-PACs significantly decreased the secretion of IL-8 and CCL5 at all concentrations tested in a dose-dependent fashion. Decreased secretion of IL-8 and CCL5 was not related to loss of cell viability, since AC-PACs did not show any cytotoxicity at up to 200 μg/ml (data not shown). However,
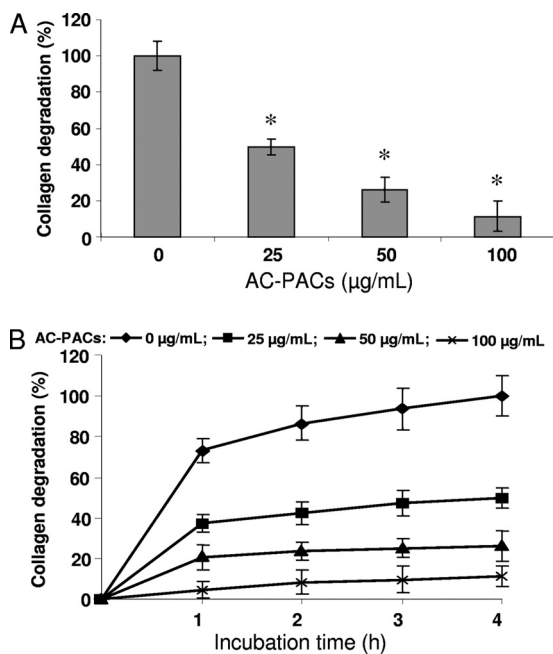
FIG. 4. Effect of AC-PACs on the degradation of type I collagen by extracellular proteases of *P. gingivalis*. Assays were run in quadruplicate, and the means ± SD from three independent assays were calculated. A value of 100% was assigned to the degradation obtained after a 4-h incubation at room temperature in the absence of AC-PACs (A). The inhibition of collagen degradation was also measured as function of time (B). *, significantly lower than the value for the untreated control ($P < 0.05$).
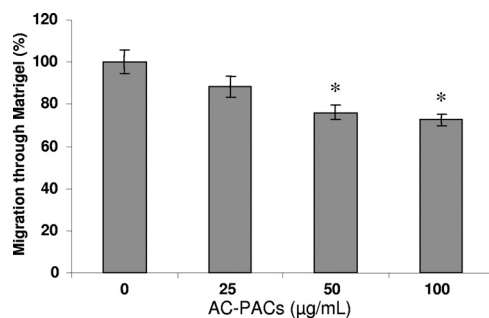
FIG. 5. Effect of AC-PACs on the migration of *P. gingivalis* through a reconstituted basement membrane model (Matrigel). ^{14}C-labeled *P. gingivalis* cells were preincubated with AC-PACs (0, 25, 50, and 100 μg/ml) for 30 min. They were then placed on the Matrigel in the top of a double-chamber system. After a 48-h incubation at 37°C under anaerobic conditions, the number of ^{14}C-labeled *P. gingivalis* organisms recovered in the lower chamber was determined by gamma counting. Assays were run in triplicate, and the means ± SD from three independent assays were calculated. A value of 100% was assigned to the amount of radioactivity counted in the absence of AC-PACs. *, significantly lower than the value for the untreated control ($P < 0.05$).

AC-PACs did not affect the secretion of IL-6. More specifically, 25, 50, and 100 μg/ml of AC-PACs reduced the secretion of IL-8 by 53.2% ± 5.3%, 84.1% ± 2.0%, and 88.3% ± 1.4%, respectively, and that of CCL5 by 63.6% ± 4.1%, 82.8% ± 2.8%, and 83.7% ± 1.4%, respectively (Fig. 6A). The relative
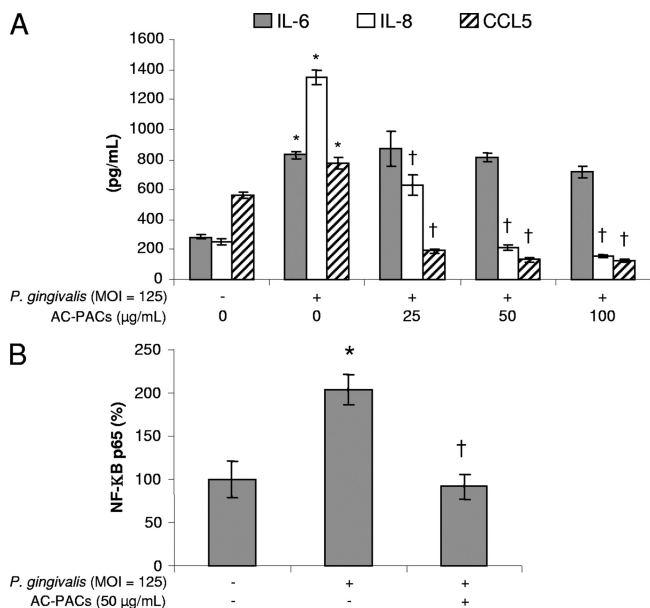
FIG. 6. Effect of AC-PACs on the *P. gingivalis*-induced inflammatory response in oral epithelial cells. Oral epithelial cells were pretreated with AC-PACs (0, 25, 50, and 100 μg/ml) for 2 h (1 h for the NF-κB p65 assay). They were then stimulated with *P. gingivalis* at an MOI of 125 for 24 h (30 min for the NF-κB p65 assay). Cell-free supernatants were collected to determine IL-6, IL-8, and CCL5 concentrations by ELISA (A). The activation of NF-κB p65 in the cellular extract was determined by an ELISA-based assay (B). Assays were run in triplicate, and the means ± SD from three independent assays were calculated. *, significantly higher than the value for the unstimulated (*P. gingivalis*) control ($P < 0.05$); †, significantly lower than the value for the untreated (AC-PACs) control ($P < 0.05$).

that they have the potential to reduce the influx of inflammatory cells to diseased sites and the amplification of *P. gingivalis*-induced inflammatory processes. We also provided evidence that AC-PACs inhibited, at least in part, the *P. gingivalis*-induced secretion of IL-8 and CCL5 by their ability to inhibit the activation of NF-κB p65, which is an activator of many cytokines and inflammatory processes (38). This is in agreement with previous studies showing that natural products, including plant polyphenols, with anti-inflammatory properties exert their effect by modulating the NF-κB pathway (7). The fact that IL-6 secretion by *P. gingivalis*-stimulated epithelial cells was not reduced by AC-PACs suggests that different cascades of kinases leading to cytokine secretion are involved and that AC-PACs may act on specific kinases.

In conclusion, the present *in vitro* study provided clear evidence that AC-PACs possess interesting therapeutic properties for the treatment of periodontal disease because of their ability to affect the etiology of periodontitis by acting on periodontopathogens and host responses. On the one hand, AC-PACs reduce the virulence properties of *P. gingivalis* by inhibiting biofilm formation, adhesion, proteinase activity, and invasiveness. On the other, AC-PACs exert anti-inflammatory activity by inhibiting the *P. gingivalis*-induced inflammatory response in human oral epithelial cells. As such, they may be of interest in the prevention and treatment of *P. gingivalis*-associated periodontal diseases. Further studies will be required to investigate the exact mechanisms by which AC-PACs neutralize the virulence properties of this bacterium.

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