Targeted Antimicrobial Photochemotherapy

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This study explores a new approach for antimicrobial therapy with light activation of targeted poly-L-lysine (pL)–chlorin $e6$ (c_{e6}) conjugates. The goal was to test the hypothesis that these conjugates between pL and c_{e6} would efficiently target photodestruction towards gram-positive (*Actinomyces viscosus*) and gram-negative (*Porphyromonas gingivalis*) oral species while sparing an oral epithelial cell line (HCPC-1). Conjugates of c_{e6} with pL (average molecular weight, 2,000) having a positive, neutral, or negative charge were prepared. Illumination with red light (λ_{max} = 671 nm) from a diode array produced a dose-dependent loss of CFU from the bacteria, under conditions that did not affect the viability of the epithelial cells. For *P. gingivalis*, the cationic conjugate produced 99% killing, while the neutral conjugate killed 91% and the anionic conjugate killed 76% after 1 min of incubation and exposure to red light for 10 min. For *A. viscosus*, the cationic conjugate produced >99.99% killing while HCPC-1 cells remained intact. The importance of the positive charge was shown by the effectiveness of c_{e6}-monoethylenediamine monoamide (a monocationic derivative of c_{e6}) in killing both bacteria. The clinically employed benzoporphyrin derivative under the same conditions killed epithelial cells while leaving *P. gingivalis* relatively unharmed. A mixture of c_{e6} with pL did not show phototoxicity comparable with that of the cationic conjugate. These results were explained by the selective uptake of the conjugates by bacteria (20- to 100-fold) compared to that by mammalian cells, while free c_{e6} showed much less selectivity for bacteria (5- to 20-fold). The data suggest that the cationic pL-c_{e6} conjugate may have an application for the photodynamic therapy of periodontal disease.

Photodynamic therapy is based on the concept that a certain photoactivatable compound, or photosensitizer (PS), can be preferentially localized in certain tissues and subsequently activated by light of the appropriate wavelength to generate singlet oxygen and free radicals that are cytotoxic to cells of the target tissue (9). Bacteria also can be killed by visible light after their treatment with an appropriate PS (3). Several studies have demonstrated that gram-positive bacteria are susceptible to photoinactivation (1, 3, 13). Although lethal photosensitization of a wide range of gram-negative bacteria has been reported elsewhere (5, 17, 33), other studies have shown that gram-negative bacteria have been resistant to photodynamic action (3, 23), unless the permeability of their outer membrane is modified (2, 22).

Periodontal disease results from the accumulation of bacterial biofilms (plaques) on tooth surfaces. Mechanical removal of the biofilms is the current method of treatment. Antiseptics and antibiotics are also used, but development of resistance in the target organisms and disruption of the oral microflora are problems associated with the use of such agents. In addition to these antibacterial therapies, the use of a PS to cause lethal photodestruction of oral bacteria has also been demonstrated elsewhere (34), indicating that photodynamic therapy could be a useful alternative in eliminating periodontopathogenic bacteria. The main problem to be overcome is devising a strategy to target the PS to the bacteria while leaving the host gingival tissue unharmed.

Many naturally occurring antibacterial peptides which all have in common inter alia a pronounced polycationic charge have been discovered (7). This is thought to be the initial factor which allows them to bind to negatively charged bacteria (30). We hypothesized that a polycationic macromolecule might effectively deliver the PS chlorin $e6$ (c_{e6}) to both gram-positive and gram-negative bacteria and allow their selective photodestruction while sparing host epithelial cells. The backbone chosen was poly-L-lysine (pL) (average molecular mass, 2 kDa). In order to investigate the effect of the charge borne by these conjugates we compared polycationic (pL-c_{e6}), neutral (pL-c_{e6}-ac), and polyanionic (pL-c_{e6}-succ) conjugates. Uptake and phototoxicity were examined in an oral epithelial cell line (HCPC-1) and two oral bacterial species: *Porphyromonas gingivalis*, a key gram-negative periodontal pathogen (26), and *Actinomyces viscosus*, a gram-positive bacterium also found in the subgingival plaque (8). In order to establish the combined role of charge and covalent conjugation, the three conjugates were compared to unconjugated c_{e6}, ce_6-monoethylenediamine monoamide (CMA) (a more cationic derivative of c_{e6}), and a mixture of pL and c_{e6}. A widely used clinical PS, benzoporphyrin derivative monoacid ring A (BPD), which is known to efficiently kill mammalian cells, was also tested.

MATERIALS AND METHODS

Preparation of pL-c_{e6} conjugates of varying charges. All reactions were carried out in the dark at room temperature and have been described previously (27). Briefly, *N*-hydroxysuccinimide ester of c_{e6} (Porphyrin Products, Logan, Utah) was prepared by reacting 1.5 equivalents of dicyclohexylcarbodiimide and 1.5 equivalents of *N*-hydroxysuccinimide with 1 equivalent of c_{e6} in dry dimethyl sulfoxide (DMSO) for 24 h and was frozen in aliquots for further use. pL hydrobromide (Sigma, St. Louis, Mo.) (average molecular weight = 2,000; 50 mg) was dissolved in dry DMSO (50 ml), and *N*-ethylmorpholine (1 ml) was added, followed by c_{e6}–*N*-hydroxysuccinimide ester (25 mg), and reacted for 24 h. After exhaustive dialysis, this gave the purified cationic conjugate (pL-c_{e6}). The DMSO solution of pL-c_{e6} was treated with an excess of acetic anhydride (100 mg dissolved in 0.5 ml of dry DMSO) to produce a neutrally charged conjugate

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(pL-c$_{e6}$-ac). In a similar fashion, the pL-c$_{e6}$ in DMSO was treated with an excess of succinic anhydride (100 mg dissolved in 0.5 ml of dry DMSO), which converted the positively charged amino groups to negatively charged carboxylic acid groups (pL-c$_{e6}$-succ). The resulting solutions of conjugates had a concentration equivalent to 0.5 to 1 mM c$_{e6}$ and were checked for purity by thin-layer chromatography (1:1 mixture of methanol and 10% aqueous NH$_4$Cl; silica gel on polyester thin-layer chromatography plates [Aldrich Chemical Co., Milwaukee, Wis.]). On this system, noncovalently bound c$_{e6}$ has an R_f value of approximately 0.5 while the conjugates remain at the origin.

Other PSs. Free c$_{e6}$ and CMA (Porphyrin Products) were dissolved in a small amount of 0.1 M NaOH solution before neutralization in phosphate-buffered saline (PBS; pH 7.4). BPD (nonliposomal) (a kind gift from QLT Phototherapeutics, Vancouver, Canada) was dissolved in a small amount of DMSO, which was diluted to give less than 0.1% DMSO in incubation medium. A 1:1 molar mixture of c$_{e6}$ and pL (degree of polymerization = 20) in PBS was also prepared.

Microorganisms. The organisms used in this study were *P. gingivalis* 381 (S. S. Socransky, Forsyth Dental Center, Boston, Mass.) and *A. viscosus* T14 (Forsyth Dental Center). Cultures were maintained by weekly subculture in Trypticase soy agar (Baltimore Biological Laboratories, Cockeysville, Md.) with 5 μg of hemin per ml, 0.3 μg of vitamin K per ml, and 5% sheep blood for *P. gingivalis* and in Trypticase soy agar with 5% sheep blood for *A. viscosus*. For experimental purposes, the organisms were grown anaerobically in a chamber with 80% N$_2$, 10% H$_2$, and 10% CO$_2$ at 35°C for 48 h; harvested by centrifugation; and resuspended in Trypticase soy broth with 5 μg of hemin per ml and 0.3 μg of vitamin K per ml. Cells were dispersed by sonication and repeated passage through Pasteur pipettes. Cell numbers were measured in a spectrophotometer (wavelength, 600 nm; 1 optical density unit equals approximately 10^9 cells/ml) in 1-ml tubes.

Oral epithelial cell line. A hamster cheek pouch cell carcinoma line (HCPC-1 [J. Schwartz, Harvard Dental School, Boston, Mass.]) was used for comparison studies (24). Cells were grown in Dulbecco's modified Eagle's medium with high glucose (Gibco, Grand Island, N.Y.) supplemented with heat-inactivated 10% fetal calf serum (Gibco), 100 U of penicillin G per ml, and 100 μg of streptomycin (Sigma) per ml. The medium was changed every 2 to 3 days, and cells were passaged weekly with trypsin-EDTA. All cells were maintained in 10-cm-diameter petri dishes with 12 ml of growth medium and kept at 37°C in a humidified, 95% air–5% CO$_2$ atmosphere.

Uptake studies. (i) Bacteria. Suspensions of the microorganisms (10^9 cells/ml) were incubated in the dark at room temperature for 1 min with 1, 5, and 10 μM c$_{e6}$ equivalent (final concentration in Trypticase soy broth) and the same concentrations for free c$_{e6}$ and CMA in triplicate. The cell suspension was centrifuged (9,000 × *g* for 1 min), the PS solution was aspirated, and bacteria were washed once by resuspending the cell pellet in 1 ml of sterile PBS and centrifuging as described above. Finally, the cell pellet was dissolved by digesting it in 1.5 ml of 0.1 M NaOH–1% sodium dodecyl sulfate for at least 24 h to give a homogeneous solution. The fluorescence of the cell extract was measured on a spectrofluorimeter (model FluoroMax; SPEX Industries, Edison, N.J.). The excitation wavelength was 400 nm, and the emission spectra of the cell suspensions were recorded from 580 to 700 nm. The protein content of the entire cell extract was then determined by a modified Lowry method (14) with bovine serum albumin dissolved in 0.1 M NaOH–1% sodium dodecyl sulfate to construct calibration curves. Results were expressed as nanomoles of c$_{e6}$ per milligram of cell protein.

(ii) Epithelial cells. Cells were trypsinized in the exponential growth phase and counted with a hemocytometer. One hundred thousand cells in 1 ml of growth medium with 10% serum were seeded into each well of 24-well culture plates. These were incubated overnight to allow cells to attach and resume exponential growth, after which time PS was added. The medium was removed and replaced with medium with 10% serum containing the conjugates, c$_{e6}$, and CMA at the concentrations described above in triplicate for 1 min. The drug solution was then aspirated from the wells, and cells were washed once with 1 ml of sterile PBS and incubated with 1 ml of trypsin-EDTA for 10 min. The resulting cell suspension was then centrifuged, the trypsin supernatant was aspirated, and the pellets were dissolved in 0.1 M NaOH–1% sodium dodecyl sulfate. The measurement of the fluorescence of the cell extract as well as the determination of the protein content was performed as described above.

Light source. The irradiation source was a 4- by 4-cm (nominal) array of 52 light-emitting diodes (Hewlett-Packard, San Jose, Calif.) mounted on a printed circuit board. The emission spectrum was determined by a spectrophotometer which had been corrected for optical spectral response. The emission peak was at 671 nm, the full-width half-maximum value of the emission was 24 nm, and 98% of the emitted light was between 635 and 695 nm, which covered the absorbance of all the PSs. The entire array was immersed in an aluminum box filled with silicone oil to provide thermal conduction to box walls, which were in turn cooled by a small fan. The maximum power available from the array was 305 mW, but the array was generally operated at about 230 mW of total power. The actual irradiance incident on the cells was determined by placing a power meter (model 200; Coherent, Palo Alto, Calif.) at the plane of the cells, measuring the power, and dividing by the active area of the meter.

For the determination of the fraction of the light falling within the absorption band of each PS, the following procedure was used. The area under the emission spectrum of the light-emitting diode array was determined numerically with 10-nm-wide steps. The absorption spectra of the PSs were measured, and the absorption maxima and the full-width half-maximum of the peaks were determined. The fraction of the light that could be absorbed by each PS was then determined by calculating the degree of overlap between the emission peak and the longest wavelength peak of the PS.

Photodynamic therapy studies. (i) Bacteria. Suspensions of bacteria (10^8/ml) were incubated in the dark at room temperature for 1 min with 5 μM c$_{e6}$ equivalent from each conjugate, the mixture of c$_{e6}$ and pL, free c$_{e6}$, and CMA and the same concentration for BPD in triplicate. Cell suspensions were centrifuged, cells were washed once with sterile PBS, and 1 ml of fresh medium was added. The bacterial suspensions were then placed in the wells of 12-well plates. All wells were exposed to light from the light-emitting diode array in the dark at room temperature. The light exposure was from below with fluences ranging from 0 to 15 J/cm^2 at an irradiance of 25 mW/cm^2. Bacteria incubated with CMA, BPD, and the mixture were exposed to only 15 J of incident light per cm^2. All plates were kept covered during the illumination in order to maintain the sterility of the culture. After illumination of the appropriate wells, serial dilutions of the contents of each well were prepared in Trypticase soy broth, and 100-μl aliquots were spread over the surfaces of blood agar plates. Survival fractions in each well were calculated by counting the colonies on the plates and dividing by the number of colonies from dark controls incubated with a PS and kept at room temperature for periods equal to irradiation times. Other controls were bacteria untreated with a PS or light but kept in plates at room temperature covered with aluminum foil during irradiation and cells exposed to light in the absence of a PS.

(ii) Epithelial cells. HCPC-1 cells (2 × 10^4) in 0.1 ml of growth medium with 10% serum were seeded in 96-well plates and cultured for 24 h until they were 70% confluent. Six wells from each plate were treated with the same concentrations of PSs as described above. After illumination, cells were incubated with fresh medium at 37°C for 24 h. Controls were the same as described above. Cell viability was determined 24 h after irradiation by the 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide microculture tetrazolium assay (20), a method of assessing the amount of dehydrogenase activity in the mitochondria of living cells. The percentage of cells surviving was determined by dividing mean 550-nm absorbances of formazan produced from treated cells by the mean absorbances from dark controls incubated with a PS and kept at room temperature for periods equal to irradiation times.

Statistics. Differences between means were analyzed for statistical significance by Student's *t* test.

RESULTS

Preparation of conjugates. The pL chains employed had an average molecular weight of 2,000, i.e., an average degree of polymerization of 20 lysine residues. Assuming quantitative recovery from dialysis, the spectrophotometric measurements showed that an average of one c$_{e6}$ molecule was present per pL chain, representing a substitution ratio of 5%. This meant that the average molecular weights of the conjugates were as follows: pL-c$_{e6}$, 2,600; pL-c$_{e6}$-succ, 4,500; pL-c$_{e6}$-ac, 3,400. The absorption spectra in 0.1 M NaOH–1% (wt/vol) sodium dodecyl sulfate and chemical structures of c$_{e6}$, CMA, and pL-c$_{e6}$ are shown in Fig. 1. The absorption spectra of conjugates were characterized by a Soret band maximum at 404 nm and a long-wavelength maximum at 664 to 666 nm. For free c$_{e6}$ and CMA, the long-wavelength maximum was 654 nm.

Uptake studies. The uptake of c$_{e6}$ from the conjugates and unconjugated c$_{e6}$ was increased on a dose-dependent basis, as Fig. 2a shows for *P. gingivalis*. Selectivity was pronounced for the cationic conjugate, which gave the highest uptake, followed by the anionic and neutral conjugates, CMA, and free c$_{e6}$ (Fig. 2a). Similar results were obtained for *A. viscosus* (Fig. 2b). At all concentrations, bacteria took up two to four times more c$_{e6}$ from the cationic conjugate than that delivered by the anionic or neutral conjugate ($P < 0.001$). Both bacteria took up significantly more c$_{e6}$ from all the conjugates than from the unconjugated molecules CMA and c$_{e6}$. *A. viscosus* took up 1.3 to 2 times more c$_{e6}$ from the three conjugates and free c$_{e6}$ than did *P. gingivalis* (Fig. 3). The uptake of c$_{e6}$ from the cationic conjugate by *A. viscosus* was almost double that of *P. gingivalis* ($P < 0.001$) at all concentrations. At all concentrations of the three charged pL conjugates, bacteria took up 20 to 100 times more c$_{e6}$ than did HCPC-1 cells (Fig. 3).

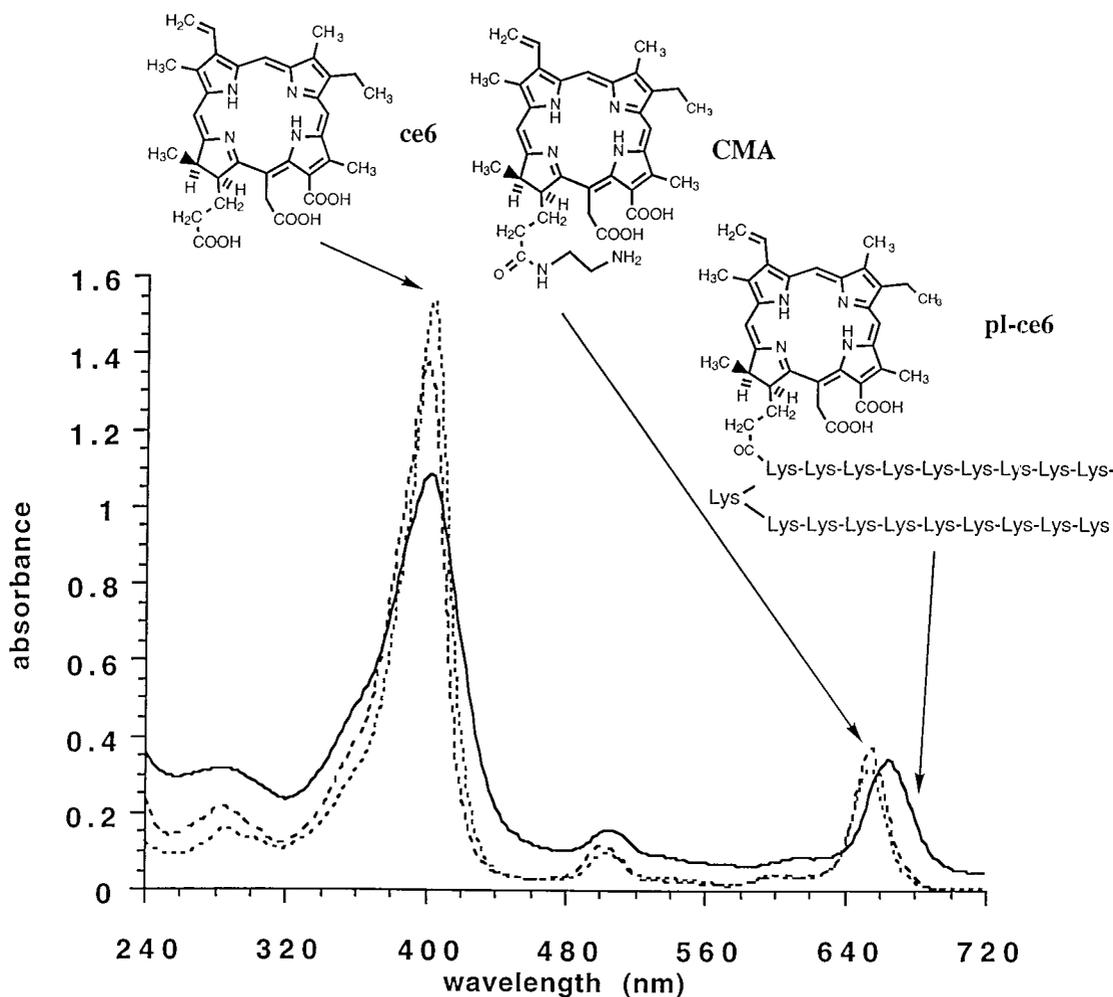For HCPC-1 cells, the cationic conjugate gave the highest

FIG. 1. UV-visible absorption spectra and chemical structures of c_{e6}, CMA, and pL-c_{e6} in 0.1 M NaOH–1% sodium dodecyl sulfate.

uptake, followed by CMA, the anionic and neutral conjugates, and c_{e6} (Fig. 2c). The uptake of c_{e6} from the cationic conjugate was 2 to 2.5 times higher than that delivered from the other PSs at all concentrations ($P < 0.001$).

Light absorbed by the PS. Table 1 gives the overlap between the absorption spectra of the PSs and the emission spectrum of the light source, which shows the percentage of the light absorbed by each PS during irradiation. The conjugates showed the highest relative absorbed light dose, followed by BPD, c_{e6}, and CMA.

Photodynamic treatment. Both *P. gingivalis* and *A. viscosus* were unaffected (reduction of viability was less than 15%) after 15 J of illumination per cm² alone or by 1 min of incubation with a 5 μM concentration of all PSs in the dark, with the exception of BPD, which showed a remarkable dark toxicity of 72% on *A. viscosus*. There was no reduction in the viability of HCPC-1 cells after their exposure to light alone or after their incubation with a 5 μM concentration of any of the PSs in the dark. The fluence values in the phototoxicity experiments have been corrected for the relative effective absorption of photons by the long-wavelength peak of the PS. The phototoxic effects of the three pL-c_{e6} conjugates and free c_{e6} on *P. gingivalis* and *A. viscosus* for increasing light doses are shown in Fig. 4. The phototoxicity was fluence dependent. The cationic conjugate gave the highest phototoxicity towards both bacteria, followed

by the neutral conjugate. The anionic conjugate and free c_{e6} gave intermediate levels of phototoxicity. Table 2 shows the photodynamic effects of all PSs on *P. gingivalis*, *A. viscosus*, and HCPC-1 cells. The cationic conjugate was the most potent of these PSs by reducing the viability of *P. gingivalis* and *A. viscosus* by 99 and >99.99%, respectively. Epithelial cells remained intact after photodynamic therapy with all PSs, except for BPD, which reduced their viability about 90% (Table 2).

The relative phototoxicities per mole of c_{e6} taken up per cell for *P. gingivalis* and *A. viscosus* are shown in Fig. 5. These numbers were calculated by taking the reciprocal of the survival fraction (Table 2) and dividing it by the number of nanomoles of c_{e6} taken up per milligram of cell protein. They are expressed relative to the values for *A. viscosus* and free c_{e6}. It can be seen that the cationic conjugate was the most potent PS, followed by CMA. The exact value for the phototoxicity of the cationic conjugate towards *A. viscosus* could not be estimated but was >99.99%.

DISCUSSION

This study tested the hypothesis that polymeric conjugates between pL and c_{e6} might selectively target bacteria for photodestruction. The effect of varying the charge of the conjugates from cationic through neutral to anionic on the selectivity
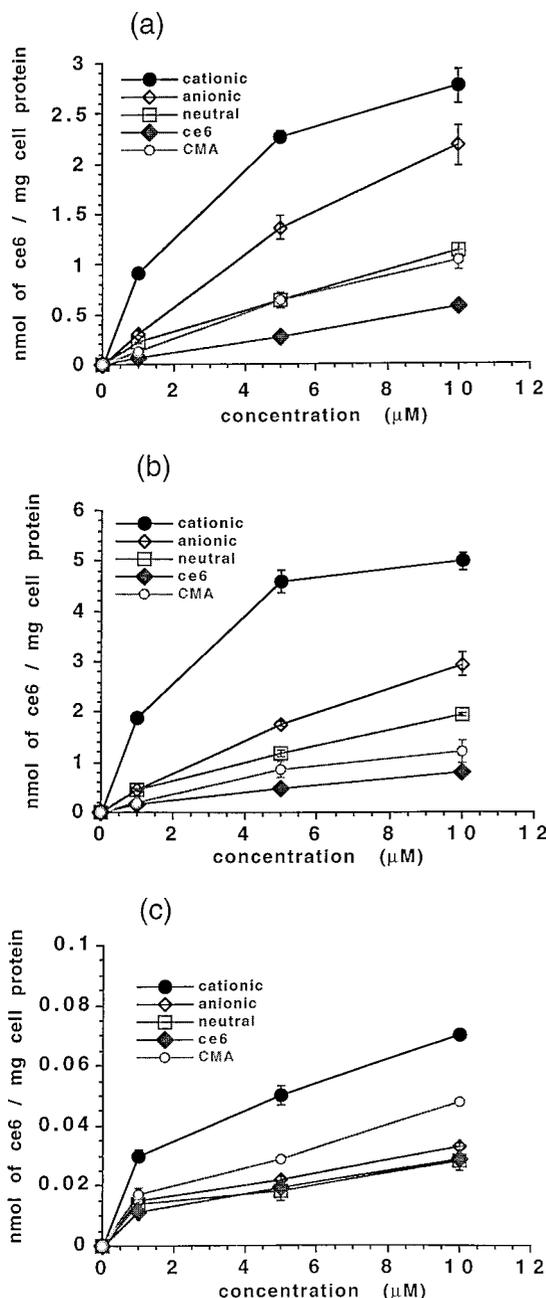
FIG. 3. Uptake of pL-c_{e6} conjugates, free c_{e6} and CMA by *P. gingivalis*, *A. viscosus*, and HCPC-1 cells. Bacteria and epithelial cells were incubated for 1 min with 5 μM c_{e6} equivalent of each conjugate. Each bar is the mean of values obtained from three tubes, and data are representative of duplicate experiments. Values are expressed as means ± standard errors of the means.

FIG. 2. Uptake of pL-c_{e6} conjugates, free c_{e6}, and CMA by *P. gingivalis* (a), *A. viscosus* (b), and HCPC-1 cells (c). Bacteria and epithelial cells were incubated for 1 min with 1, 5, and 10 μM c_{e6} equivalent of each conjugate, and c_{e6} uptake was expressed as nanomoles of c_{e6} equivalent per milligram of cell protein. Each bar is the mean of values obtained from three tubes, and data are representative of duplicate experiments. Values are expressed as means ± standard errors of the means.

they exhibited towards two oral bacteria of the subgingival plaque and an oral epithelial cell line was studied. We specifically wished to determine whether a polycationic conjugate which emulated well-known natural cationic antibacterial peptides would be effective for delivering PS to gram-negative bacteria.

Conjugation of c_{e6} molecules to pL promotes the uptake of c_{e6} by oral bacteria and HCPC-1 cells, and this increased uptake occurs in a concentration-dependent manner. The cat-
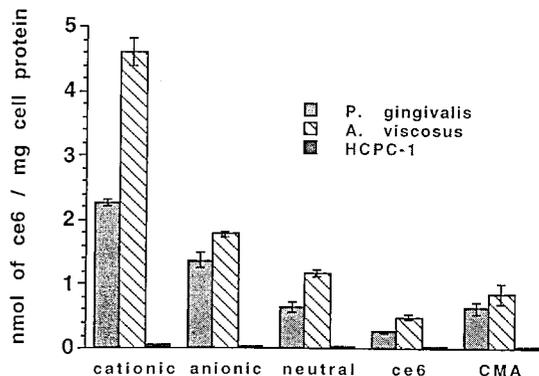
ionic conjugate showed the highest uptake for both *P. gingivalis* and *A. viscosus*. Neither species of oral bacteria accumulated significantly more c_{e6} from the cationic conjugate at 10 μM than at 5 μM. This similarity of uptake suggested a saturation effect, and therefore the 5 μM concentration was used in subsequent photosensitization studies. The enhanced uptake of c_{e6} from the cationic conjugate could be due to the binding of the positively charged pL-c_{e6} to the outer membrane of *P. gingivalis* as well as to the phosphate groups of the wall teichoic acids of *A. viscosus*, both of which bear a strong negative charge (29). HCPC-1 cells accumulated 30 to 100 times less c_{e6} from the cationic conjugate than did bacteria, although the plasma membrane of malignant epithelial cells also bears an overall net negative charge, which is greater than that of normal epithelial cells due to overexpression of polysialic acid residues (11).

An increasing array of small naturally occurring antibacterial peptides have been discovered (7). These have been found in mammals, fish, insects, and invertebrates (6). Examples of this class include histatins, defensins, magainins, cecropins, and tachyplasins. The mechanism of action of these peptides is being intensively investigated, but a common factor in all the structures is the polycationic charge due to lysine, histidine, and arginine residues in the amino acid sequence (7). Although tertiary structures such the alpha-helix are thought to play a role in their selective cytotoxicity towards bacteria (10), the polycationic charge borne by these polypeptides is probably responsible for their initial binding to bacteria.

Photosensitization of microorganisms was carried out in

TABLE 1. Overlap between the absorption spectra of the PSs and the emission spectrum of the diode array

PS	Absorption peak (half peak width) (nm)	Extinction coefficient[a]	% Overlap with light emission	Light dose[b]
c_{e6}[c]	658(22)	30,000	35	79.5
CMA	658(24)	28,000	37	78
Conjugate	662(28)	20,000	66	100
BPD	687(19)	50,000	24	91

[a] Measured in 0.1 M NaOH–1% sodium dodecyl sulfate.
[b] The relative effective light dose absorbed by each PS is defined as extinction coefficient × overlap with diode emission in percent and was expressed as percentage of that absorbed by the conjugates.
[c] No difference in the spectrum was observed when c_{e6} was mixed with pL.
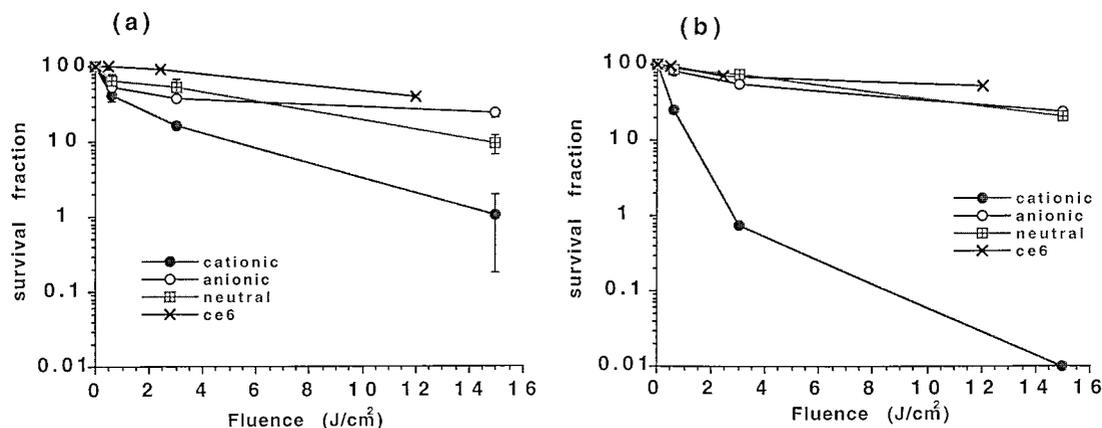
FIG. 4. Phototoxicity of *P. gingivalis* (a) and *A. viscosus* (b) after incubation with 5 μM c_{e6} equivalent from each conjugate and free c_{e6} for 1 min followed by treatment with red light and colony-forming assay. Each point is the mean of values obtained from three wells, and data are representative of duplicate experiments. Values are expressed as means ± standard errors of the means.

complete culture medium after exposure to the PS for only 60 s; effective killing is more challenging under these conditions than under conditions of incubation in PBS in the presence of the PS. The use of solutions such as PBS for both the incubation and the illumination of bacteria allows much easier photoinactivation than do the protective effects of complete medium. For the removal of the weakly bound PS prior to illumination, only one washing in PBS was used, as extensive washings can damage the membrane barrier of gram-negative bacteria (21). Consistent with this concept, it has been shown that only one washing was enough to remove more than 60% of the originally bound cationic water-soluble zinc phthalocyanine from *Escherichia coli* (18). The amount of the PS removed after four washings was 80% (18). The same authors showed that there was no substantial decrease in the degree of bacterial killing with light after several washings, suggesting that it was the tightly bound PS rather than the weakly bound PS which was involved in the photosensitization.

The pronounced photodynamic effect of the cationic conjugate on *A. viscosus* may be due to the electrostatic attraction between the conjugate and the negatively charged membrane of the bacterium. This hypothesis is supported by data regarding the phototoxicity of a mixture of pL (positively charged) and c_{e6} as well as of free c_{e6} on *A. viscosus*. A reduction of 60% in survival of *A. viscosus* was achieved after treatment with visible light of 12 J/cm² with free c_{e6} (Table 2). However, c_{e6} did not have any photodynamic effect when the mixture was used (Table 2). It is likely that pL interacts with the negatively charged cell wall and blocks the interaction of c_{e6} with bacteria, so that after washing most of the PS has been removed.

We have previously shown that both epithelial and endothelial cells accumulate sufficient c_{e6} from charged pL conjugates to enable efficient photoinactivation to take place (27). However, in mammalian cells this process of uptake is time dependent due to the necessity of these macromolecules being actively internalized, and the sparing of HCPC-1 cells in the present study is due mainly to the very short incubation time. This short incubation time reduced the total uptake of the HCPC-1 cells and also meant that the amount of c_{e6} which did bind to the cells was likely to be at the plasma membrane rather than internalized. If the PS is localized at the plasma membrane, then it will absorb light and transfer its energy to molecular oxygen, forming singlet oxygen, which has a mean diffusion distance of 100 to 200 nm (19). Because mammalian

epithelial cells have a volume often 2,000 times greater than that of bacteria (29), active oxygen species generated at the surface are very much less likely to be able to diffuse to a more sensitive intracellular location than those in the case of bacteria. We have previously shown that in epithelial cells after 6 h of incubation the cationic conjugate had the lowest relative phototoxicity compared to neutral and anionic conjugates (27). This situation is in complete contrast to the present study, where the cationic conjugate has by far the highest relative phototoxicity in bacteria after 1 min of incubation.

In the case of *P. gingivalis*, other mechanisms may operate, as this interaction was weaker and the structure of the outer membrane is more complex. The entire problem of photodynamic inactivation of gram-negative bacteria has been comprehensively reviewed elsewhere (12), and the structure of the lipid layers of the outer membrane is thought to be responsible for their remarkable resistance to conventional anionic PS (22). Antibacterial photodynamic activity on gram-negative species has been achieved in the presence of membrane-disorganizing polycations such as polymyxin B nonapeptide (21). pLs are among the polycations which bind to the anionic sites of lipopolysaccharide. This binding may weaken the intermolecular interactions of the lipopolysaccharide constituents, dis-

TABLE 2. Photoinactivation of *P. gingivalis*, *A. viscosus*, and HCPC-1 cells[a]

PS	% Survival for organism or cell line		
	P. gingivalis	*A. viscosus*	HCPC-1
Cationic conjugate	1.1 ± 0.9	<0.01	102 ± 2.1
Anionic conjugate	23.9 ± 3.3	24.3 ± 3.4	127 ± 2.9
Neutral conjugate	9.3 ± 2.6	21.2 ± 1.5	108 ± 4.2
Free c_{e6}	39.6 ± 2.3	52.9 ± 5.9	126 ± 2.7
CMA	8.1 ± 1.8	2 ± 0.1	100 ± 2.5
BPD	81.1 ± 9.4	7.6 ± 7.6	8.8 ± 0.7
Mixture (pL and c_{e6})	8.1 ± 1.8	2 ± 0.1	100 ± 2.5

[a] The percent survival of bacteria was assayed by the colony-forming assay following 1 min of treatment with PS and exposure to 15 J/cm². The viability of HCPC-1 cells was assayed by the microculture tetrazolium assay. Surviving bacteria and epithelial cells were expressed as a percentage of controls receiving PS in the dark out of the incubator. Values for bacteria are the means from three wells, whereas for HCPC-1 cells values are means from six wells, and data are representative of duplicate experiments (± standard errors of the means).
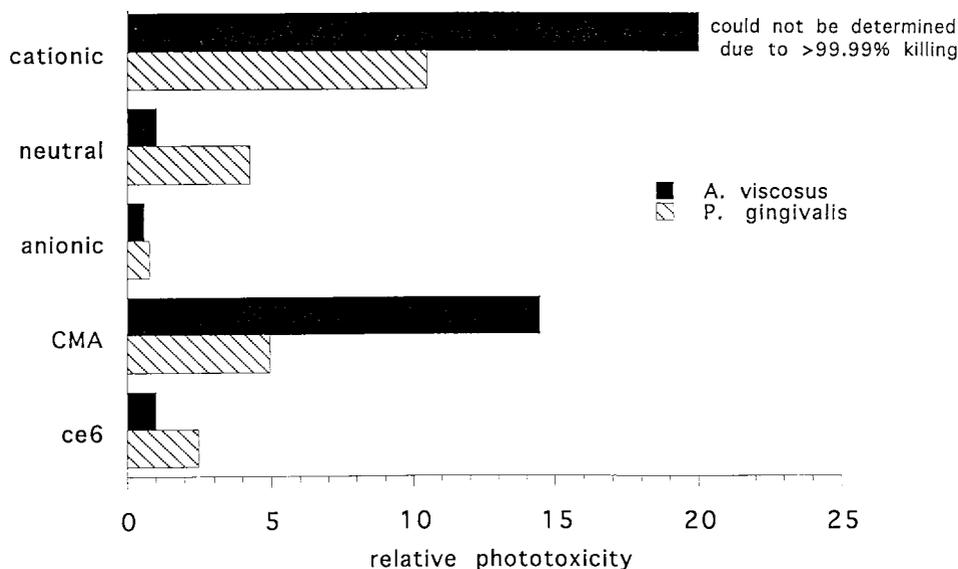
FIG. 5. Relative phototoxicity (at 15 J/cm^2) of c_{e6} delivered by the conjugates, free c_{e6} and CMA per mole of c_{e6} taken up per cell for *P. gingivalis* and *A. viscosus*. The phototoxicity is expressed relative to the values for *A. viscosus* and free c_{e6}.

organize the structure, and render it permeable to drugs (30) by enabling them to cross the outer membrane. The effect of a pL with 20 lysine residues (similar to that used in this study) on smooth *Salmonella typhimurium* after a short treatment (10 min) was a rapid release of 20 to 30% of the lipopolysaccharide from the outer membrane and the subsequent sensitization of the bacteria to the anionic sodium dodecyl detergents (31). The same authors also showed that this polymer sensitized smooth *E. coli* and *S. typhimurium* strains to hydrophobic antibiotics by a factor of 100 or more (32). In both studies, the polymer was not found to be bactericidal. It is possible that the cationic conjugate begins its action by electrostatic interaction with the negatively charged groups in the lipopolysaccharide of *P. gingivalis* and then subsequently alters the permeability of the outer membrane, and therefore the conjugate gains access to internal targets. Furthermore, the photodynamic effect of c_{e6} of *P. gingivalis* became more pronounced when the mixture with pL was used (Table 2). This suggests that the positively charged pL altered the outer membrane barrier function and helped c_{e6} to penetrate the cell. In the case of the neutral conjugate, pL seems to have the same role as above. Although the uptake of c_{e6} from the neutral conjugate by *A. viscosus* was twice that of *P. gingivalis*, the phototoxicity of the conjugate was much greater on *P. gingivalis* (Table 2). Recently, it has also been suggested that the resistance of gram-negative bacteria to photoinactivation could be related to the charge on the PS (15, 18). The gram-negative bacteria *E. coli* and *Pseudomonas aeruginosa* could be photoinactivated by illumination in the presence of a cationic water-soluble zinc pyridinium phthalocyanine for 30 min but not by illumination in the presence of a neutral tetradiethanolamine phthalocyanine or a negatively charged tetrasulfonated phthalocyanine (18). Meso-substituted cationic porphyrins were also efficient PSs of gram-negative bacteria such as *Vibrio anguillarum* and *E. coli* after incubation for 5 min (15). In another study, the authors suggested that it was the positive charge that promoted the binding of the porphyrin to the outer membrane, inducing a limited damage which favored the penetration of the PS (16). They also showed that the photosensitizing activity of cationic porphyrins toward gram-negative bacteria was inhibited by their incorporation into liposomes (16).

The uptake and photosensitization of both *A. viscosus* and *P. gingivalis* by the anionic conjugate were almost the same, and this is consistent with electrostatic repulsion between the charged sensitizer molecule and the cell membrane. CMA was the second most potent PS, after the cationic conjugate, for both bacteria (Fig. 5). This is not surprising, considering that CMA has a carboxyl group replaced by a primary amino group, thus markedly increasing its cationic character. Although it is not polycationic and cannot disrupt the gram-negative bacterial outer membrane, its small size may still allow it to penetrate the outer surface of both bacteria. BPD, a hydrophobic chlorin-like porphyrin derivative used in clinical trials, was very phototoxic on *A. viscosus* and on HCPC-1 cells (Table 2). The very strong dark cytotoxic effect of BPD on *A. viscosus* (72% reduction of survival) was a surprising finding. Because of its hydrophobicity, BPD may penetrate the cellular membrane quickly and localize in an intracellular site that is very sensitive to photodamage. The more complex structure of the outer membrane of *P. gingivalis* may prevent the diffusion of BPD into the bacterial cell. No PS except BPD led to any phototoxicity on HCPC-1 cells at any concentration tested in this study, suggesting that there is a therapeutic window whereby bacteria could be killed without damaging epithelial cells.

A number of studies have shown that oral bacteria (including *P. gingivalis*) could be killed by red light after sensitization with the cationic PS toluidine blue in pure cultures of the organisms (35), in biofilms (5), or as a mixed population present in subgingival plaque from patients with chronic periodontitis (25). *P. gingivalis* could be photoinactivated after sensitization with toluidine blue under various environmental and physiological conditions (4). Lethal photosensitization of *Streptococcus sanguis*, a major plaque-forming organism, was also possible with toluidine blue at sensitizer concentrations and light doses that did not affect the viability of oral keratinocytes and oral fibroblasts (28). The disadvantage of this approach is the dark blue staining of the oral mucosa obtained with this dye.

As a whole, our results suggest that the increased uptake and the pronounced phototoxicity of the cationic c_{e6} conjugate on *P. gingivalis* and *A. viscosus* depend on the charge borne by the conjugate. Based on the data in the present study, we propose that the disorganizing action of the 20-residue pL chain leads

to increased penetration of the PS through the outer membrane of gram-negative bacteria, which significantly contributes to the enhanced photodynamic effect of the cationic conjugate on *P. gingivalis*. The results clearly showed that the viability of oral malignant keratinocytes was not affected after their incubation with the cationic conjugate for 60 s and exposure to visible light. Further studies in an animal model of periodontitis, where the PS will be introduced into the dental pocket, followed by red light illumination via an optical fiber, will show whether the cationic conjugate will have an application for therapy of periodontal disease.

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