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The anti-endotoxic effects of the KSL-W decapeptide on *Escherichia coli* O55:B5 and various oral lipopolysaccharides

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Background and Objective: Host responses following the recognition of bacterial lipopolysaccharide can range from acute inflammation to septic shock. The aim of this study was to evaluate the ability of the KSL-W decapeptide to bind to and block the endotoxic effects of lipopolysaccharide.

Material and Methods: An enzyme-linked immunosorbent assay-based binding assay using fluorescently labeled KSL-W to detect adsorbed *Escherichia coli* O55:B5 lipopolysaccharide was employed. A commercially available recombinant Factor C lipopolysaccharide detection assay, hemagglutination of rabbit erythrocytes as well as E-selectin expression in human umbilical vein endothelial cells were used to assess the anti-endotoxic effects after KSL-W exposure to *E. coli* lipopolysaccharide as well as to oral lipopolysaccharide samples.

Results: Lipopolysaccharide-binding assays using *E. coli* O55:B5 lipopolysaccharide revealed both a higher maximal binding range (532–713 μM) and a half-maximum binding concentration (70–185 μM) for the KSL-W peptide when compared with its analog control. Significant inhibition of E-selectin expression in human umbilical vein endothelial cells ($p < 0.0001$) as well as hemagglutination of rabbit erythrocytes occurred after the interaction of KSL-W with *E. coli* lipopolysaccharide. Recombinant Factor C enzyme detection inhibition revealed dose-dependent inhibition values ranging from 1.0–51.8 μM, which were dependent upon the type of lipopolysaccharide sample tested.

Conclusion: These results demonstrate that for the concentrations tested, the KSL-W decapeptide was nontoxic to mammalian cells and could bind to and block the host recognition and response towards enteric, as well as oral, lipopolysaccharide samples.

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Recognition of bacterial cellular components is a key innate defensive feature of a variety of plant and animal species (1,2). A classic example of one such component is bacterial lipopolysaccharide. Lipopolysaccharides are amphiphilic molecules that make up a large majority of the outer membrane surface of virtually all gram-negative bacteria. Once expressed on the surface of the bacterium or shed as a result of cell lysis, specific molecular patterns (contained within segments of the lipopolysaccharide molecule) are then available for recognition by an

assortment of host cells or proteins (3–8). This recognition event has been examined extensively and, in turn, can lead to a series of sequential inflammatory host events ranging from localized acute inflammation to systemic endotoxic shock (4,9,10).

Between 500–800 synthetic and naturally occurring antimicrobial peptides have been reported and provide an initial and effective defensive front against foreign components and microbial invasion (2,11–13). The vast majority of these antimicrobial peptides have a broad spectrum of biologic activity that is linked to the chemical and physical properties of the peptides themselves. For example, structure–activity relationship studies have determined that peptide length (> 20 peptides), three-dimensional shape (α-helix and β-sheet), as well as charge potential, assist in microbial membrane adherence and lytic activity of the antimicrobial peptide (11,12,14,15). However, over the last 5–10 years of antimicrobial research, increasing emphasis has been placed on identifying critical internal sequences in an attempt to reduce the size of these peptides while still maintaining their inherent antibacterial activity (15) and/or their ability to bind to and neutralize microbial components such as lipopolysaccharide (16–18).

Previous laboratory studies have demonstrated significant antimicrobial activity of a short antimicrobial peptide (KSL) on a wide range of bacteria and fungi (19–21), as well as its ability to disrupt oral biofilm growth (22). Recently, KSL-W, an analog of KSL, was synthesized and has shown improved stability in the oral cavity, safety in the gastrointestinal tract and bactericidal activity (23). However, what has yet to be established is the ability of this small, positively charged KSL-W peptide to interact with and potentially affect microbial products such as lipopolysaccharide. Therefore, in this study we employed the use of enzyme-linked immunosorbent assay (ELISA), commercially available lipopolysaccharide detection systems as well as specific host cell assays to determine the ability of this peptide to bind to and alter the host recognition and response towards lipopolysaccharide from both oral and enteric bacteria.

Material and methods

Lipopolysaccharide

Lipopolysaccharide from *Escherichia coli* O55:B5 was purchased from Sigma (Sigma-Aldrich, St Louis, MO, USA). Lipopolysaccharide from *Porphyromonas gingivalis* ATCC 33277, *Prevotella intermedia* ATCC 25611, *Fusobacterium nucleatum* ATCC 10953, *Aggregatibacter actinomycetemcomitans* (formerly *Actinobacillus actinomycetemcomitans*) ATCC 43718 was obtained from lyophilized whole cells via the TRI-reagent extraction method (24) incorporating the following modifications. Briefly, 50 mg of lyophilized whole bacterial cells were suspended in 1 mL of TRI-reagent (Tri-reagent, MRC Inc., Cincinnati, OH, USA). Complete mixing of this initial suspension is critical and was accomplished by vortexing and sonication as needed. Samples were allowed to incubate for 15–30 min at room temperature to complete the homogenation process. After incubation, 200 μL of chloroform was added and the mixture was vortexed again and subjected to further incubation at room temperature (24.4°C) for 15–30 min. Aqueous and organic phase separation were accomplished by centrifugation, as described previously (24). Only the aqueous phase, which contains the lipopolysaccharide–lipid A complex, was removed and saved. Repeated extractions of lipopolysaccharide from the organic phase were conducted by adding endotoxin-free distilled water to the original remaining organic phase and repeating the vortexing, incubation and centrifugation procedures as before. All additional aqueous phase separation products were removed and combined with the first aqueous extraction. These pooled extractions were snap frozen, stored at −80°C overnight and then lyophilized (Freezone 4.5; Labconco Corp., Kansas City, MO, USA). Before use, these lipopolysaccharide preparations were purified using the cold $MgCl_2$–ethanol purification procedure (25).

KSL-W antimicrobial decapeptide, analog and fluorescent bioconjugates

The KSL-W (KKVVFWVKFK-NH_2) peptide (+ 4 charge, 60% hydrophobic residues) was synthesized using an automatic peptide synthesizer (Model 90; Advanced ChemTech, Louisville, KY, USA) employing standard solid-phase procedures and 9-fluorenylmethoxycarbonyl chemistry (22). Purity was determined as previously described by our laboratory (21). Both the fluorescently labeled KSL-W and the KSL-W analog (A6) (VVVFWVVVF-NH_2) HiLyte Fluor 488 bioconjugate, used for binding assays in this study, were manufactured commercially (AnaSpec, San Jose, CA, USA). The net positive ionic charge was removed from the (A6) analog by replacing the positively charged lysine residues (K) with neutral valine (V).

Lipopolysaccharide-binding ELISA assay

Lipopolysaccharide obtained from from *E. coli* O55:B5 [50 μg/mL of lipopolysaccharide (100 μL/well)] was first adsorbed overnight onto a flat-bottom, Hi-White 96-well ELISA plate (PerkinElmer, Shelton, CT, USA) using the chloroform–ethanol evaporation method, as described previously (26,27). Verification of adsorbed *E. coli* O55:B5 lipopolysaccharide, using a commercially obtained lipopolysaccharide-Alexa-Fluor 568 conjugate (Molecular Probes; Invitrogen, Carlsbad, California, USA), and non-specific binding of the KSL-W and KSL-W (A6) Hi-Lyte 488 conjugates were determined through experimental optimization procedures using a luminescence spectrometer set to fluorescence (Perkin Elmer LS55 Luminescence spectrometer; FL WINLAB Software V4.0; Perkin Elmer Instruments, Shelton, CT, USA). After optimization, an ELISA-based binding assay was performed according to the protocol described by Li *et al.* (16) with the following modifications. After overnight incubation with optimal levels of test lipopolysaccharide, the plate was washed four times with

300 μL/well of a standard wash solution (phosphate-buffered saline containing 0.05% Tween 20), then a blocking solution containing 2% bovine serum albumin (2% bovine serum albumin in phosphate-buffered saline containing 0.05% Tween 20) was applied and the plate was incubated at 37°C for 1 h. The blocking solution was removed by washing the plate twice, then increasing concentrations of the KSL-W-Fluorophore 488 conjugate (50 μL/well) were applied and allowed to interact with the adsorbed lipopolysaccharide for 3 h at room temperature. Unbound peptide was removed by gently washing the plate seven times with 300 μL/well of the washing solution. Next, 100 μL of endotoxin-free distilled H_2O was added to each well and fluorescence was measured using a luminescence spectrometer (PerkinElmer LS55; Perkin Elmer Instruments, Shelton, CT, USA) at excitation and emission values of 503 and 528 nm, respectively. Values expressed were correlated to the amount of lipopolysaccharide present and bound KSL-W bioconjugate.

Lipopolysaccharide neutralization assay

To test the ability of KSL-W peptide to interact with and neutralize lipopolysaccharide, we employed the use of a commercial lipopolysaccharide-specific assay (PyroGene; Cambrex Bio Science Wakersville, Inc., Walkersville, MD, USA), as recently described by Li *et al.* (16). Briefly, the assay is based on the ability of a recombinant clotting enzyme to become activated by lipopolysaccharide binding, then interact with an added fluorogenic substrate producing a fluorescent signal proportional to the lipopolysaccharide concentration. In this assay, each individual lipopolysaccharide sample tested was first optimized to achieve assay values within the manufacturer's standardized curve value for each assay. Next, varying starting amounts of the KSL-W peptide were applied to equal volumes of a fixed concentration of either *E. coli* O55:B5 lipopolysaccharide or various prepared oral lipopolysaccharide samples in sterile, endotoxin-free borosilicate tubes and allowed to interact at 37°C for 1 h. Next, 100 μL of this mixture was applied to a sterile microtitre plate (Hi-White Microtitre plates; Perkin-Elmer), followed by the addition of 100 μL of freshly reconstituted recombinant Factor C reagent, buffer and fluorogenic substrate mixture, according to the manufacturer's instructions. After incubation for 1 h at 37°C, fluorescence was measured (440 nm emission) and standardized against the blank readings taken at time zero for each sample. Percentage inhibition was calculated as the value obtained from [(Blank corrected lipopolysaccharide standard value)−(Blank corrected sample value)]/(Blank corrected lipopolysaccharide standard value) × 100 for each individual sample. To compare results between different lipopolysaccharide samples, the concentration of the KSL-W peptide required to achieve 50% inhibition of the recombinant Factor C activity value was determined from calculated extrapolation results of recorded data values obtained within the linear inhibition activity range.

Human umbilical vein endothelial cells E-selectin assay

To assess whether KSL-W binding to lipopolysaccharide would alter E-selectin activation in human umbilical vein endothelial cells, an ELISA-based, human umbilical vein endothelial cell E-selectin assay was utilized. First, assay optimization experiments were performed to determine the linear range of E-selectin stimulation that occurred, using the *E. coli* O55:B5 lipopolysaccharide as a stimulation control. Then, a lipopolysaccharide concentration (10 ng/mL) that was within this linear stimulatory range of E-selectin expression was co-incubated with increasing amounts of KSL-W in sterile, endotoxin-free borosilicate tubes and allowed to interact at 37°C for 1 h. The E-selectin assay, as well as the maintenance of human umbilical vein endothelial cells, were performed as previously described (28). Interleukin-1β is known to produce a strong E-selectin response in human umbilical vein endothelial cells (29) and was also used as an additional positive control for this assay. Cell viability studies to determine the extent of toxicity from KSL-W exposure were performed by comparing interleukin-1β-induced E-selectin expression with the Trypan blue exclusion assay. In these assays, separate cellular suspensions of human umbilical vein endothelial cells were exposed to Trypan blue after pre-incubation with increasing concentrations of KSL-W. After 3 min of exposure to Trypan blue, cell suspensions were placed on a hemocytometer and observed under a light microscope. Viable cell percentage calculations were made by dividing the total number of viable cells per millilitre of aliquot by the total number of cells per millilitre of aliquot multiplied by 100. Next, to determine the maximal KSL-W concentration before loss of cellular viability, cell perturbation studies were correlated to the interleukin-1β expression values, performed in additional human umbilical vein endothelial cell suspensions after co-incubation with the same increasing concentrations of KSL-W.

Hemagglutination assay

It has been well reported that lipopolysaccharide from various bacteria can induce hemagglutination in erythrocytes (30–33). We therefore tested the ability of the KSL-W peptide to block *E. coli* O55:B5 lipopolysaccharide-induced hemagglutination. In this assay, rabbit erythrocytes were exposed to this lipopolysaccharide alone or combined with KSL-W, as described above. Erythrocyte preparation and the hemagglutination assay were performed as described previously with the following modifications (34,35). Briefly, rabbit erythrocytes [rabbit blood (alsevers); PML Microbiologicals, Wilsonville, OR, USA] were washed twice in 0.01 M phosphate-buffered saline, pH 7.4 (Sigma) and resuspended in NCN buffer (150 mM sodium chloride, 3 mM sodium citrate) to make a 2% (v/v) suspension. Then, 50 μL of sample solutions [increasing concentrations of *E. coli* O55:B5 lipopolysaccharide combined with equal volumes of

phosphate-buffered saline, increasing lipopolysaccharide combined with equal volumes but a constant concentration of the KSL-W peptide (500 μg/mL), or experimental controls] were incubated for 1 h at 37°C and then added to 50 μL of washed erythrocyte solution in a V-bottom 96-well microtitre plate (Costar #3896; Corning, NY, USA). Samples were allowed to interact with this erythrocyte solution within the microtitre plate wells incubated at 37°C for 30 min while subjecting the plate to slight agitation. Plates were then removed from incubation and stored overnight at 4°C. The following day, positive agglutination results were scored visually and identified as those wells containing a thin to thick but adherent layer of agglutinated red blood cells on the sides of the wells with no significant red blood cell lysis. Experimental controls used were as follows: whole cells washed and resuspended in phosphate-buffered saline buffer from *P. intermedia* strain #17 (optical density at 600 nm = 1.5) were used as the positive control (34) and phosphate-buffered saline alone, which formed a dense spot of erythrocytes at the bottom of the well without any adherence layer, was used as the negative control for each assay.

Statistical analysis

Significance of differences between stimulatory control values and KSL-W peptide co-applied were analysed using the unpaired Student's *t*-test. The Bonferrroni correction was applied to reduce the potential for increased experimental wide alpha error after *t*-test analysis of multiple groups. Nonlinear regression (one-site binding hyperbola) was used to analyse a saturation isotherm and to determine maximal binding and half-maximal binding values.

Results

KSL-W decapeptide binds to lipopolysaccharide

The ability of KSL-W to bind to *E. coli* O55:B5 lipopolysaccharide was determined and compared with a control peptide, designated as KSL-W decapeptide analog (A6), that contained a charge-altered (positive to neutral replacement) sequence. In these assays, the KSL-W peptide showed a dose response and saturation affinity for adsorbed *E. coli* O55:B5 lipopolysaccharide. Nonlinear regression was used to display the saturation binding curve for the KSL-W peptide by determining the appropriate curve fit of the observed data points. Analysis revealed that the maximal binding concentration range for KSL-W was 531.5–713.0 μM with a half-maximal binding concentration range of 70.4–184.6 μM. By contrast, the control peptide KSL-W (A6) did not significantly bind to lipopolysaccharide under identical increasing concentration conditions (Fig. 1). These results are in agreement with previous reports identifying the importance of positive charge and lipopolysaccharide binding (11,12,36–39).

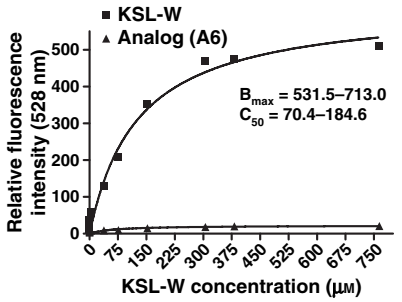

Fig. 1. KSL-W peptide and binding capacity for *Escherichia coli* O55:B5 lipopolysaccharide compared with the KSL-W analog (A6). Increasing amounts of fluorescently labeled KSL-W peptide (+4 charge), as well as the neutral KSL-W analog peptide (A6), were added to determine binding affinity to the adsorbed lipopolysaccharide. Nonlinear regression analysis was employed to determine the maximal binding concentration range as well as the concentration to achieve half-maximal binding for KSL-W. The results from these experiments revealed that the KSL-W peptide bound to the lipopolysaccharide in a dose-dependent manner as compared to the analog (A6) control, with binding saturation occurring at or above a KSL-W concentration of 530 μM. The data presented are representative of more than three separate experiments. B_{max}, maximal binding; C_{50}, half-maximal binding.

KSL-W inhibits *E. coli* lipopolysaccharide-induced recombinant factor C activity

To determine whether KSL-W binding to *E. coli* O55:B5 lipopolysaccharide would inhibit recognition and potentially its endotoxic properties, we first employed the use of a commercially available recombinant Factor C fluorometric assay. Preliminary optimization experiments resulted in routine preparation and use of the 1 enzyme unit (1 EU/mL) commercial standard for the lipopolysaccharide assay control concentration in all recombinant Factor C inhibition assays. Percentage inhibition calculations from these experiments revealed that KSL-W was able to neutralize more than 85% of the combined control lipopolysaccharide at a starting peptide concentration of 7.6 μM (Fig. 2). A 10-fold reduction in this starting concentration of KSL-W resulted in a drop of approximately 50% in lipopolysaccharide neutralization, whereas a 10-fold increase above a 7.6 μM concentration resulted in lipopolysaccharide neutralization saturation at approximately 90–95% (Fig. 2). Calculation of the 50%

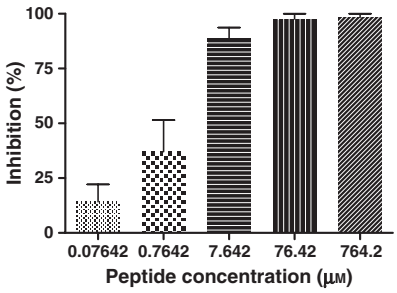

Fig. 2. Recombinant Factor C assay and neutralization of *Escherichia coli* O55:B5 lipopolysaccharide by KSL-W. Percentage inhibition of fluorescence was calculated for each concentration of the KSL-W peptide after co-incubation with the *E. coli* lipopolysaccharide sample. The results indicate that binding of KSL-W to lipopolysaccharide inhibits the lipopolysaccharide-induced recombinant Factor C fluorescent activity in a dose-dependent manner, with approximately 50% inhibition calculated within the 1.0–4.3 μM peptide concentration range. Mean results and standard deviation were generated from three separate experiments with triplicate wells.

inhibition value revealed that a starting concentration range of approximately 1.0–4.3 μM was required to achieve 50% inhibition of recombinant Factor C fluorescent activity.

KSL-W inhibits E-selectin expression of lipopolysaccharide in human umbilical vein endothelial cells

The potential of the KSL-W peptide to block the direct stimulation of E-selectin in human umbilical vein endothelial cells was also examined (Fig. 3). E-selectin expression was evaluated in human umbilical vein endothelial cells after 4 h of exposure to either *E. coli* lipopolysaccharide alone or to *E. coli* lipopolysaccharide pre-incubated with KSL-W. Similarly to previous reports using lipopolysaccharide (28,40,41), we observed consistent E-selectin responses within the human umbilical vein endothelial cells assay system that required as little as 1–100 ng/mL of *E. coli* lipopolysaccharide to yield a significant response. Optimization assays revealed that this concentration (10 ng; *E. coli* O55:B5) was in the middle of the linear stimulation range for E-selectin expression in human umbilical vein endothelial cells and was therefore used as the stimulation dose. In contrast to this, when the same concentration of *E. coli* lipopolysaccharide was pre-incubated with increasing concentrations of KSL-W, significant inhibition of E-selectin expression occurred ($p < 0.0001$; unpaired *t*-test) starting at 25 μg/mL and continuing through the 200 μg/mL KSL-W concentration range (Fig. 3). Endothelial cell viability after stimulation was checked as described previously (28) as well as by evaluating the interleukin-1β-induced E-selectin expression after co-incubation with the same increasing KSL-W concentrations, as described in the Material and methods section (data not shown).

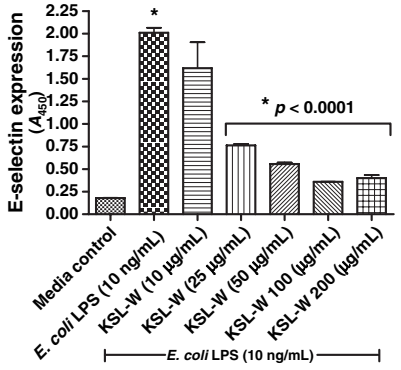

Fig. 3. Inhibition of E-selectin expression by *Escherichia coli* O55:B5 lipopolysaccharide in human umbilical vein endothelial cells. A stimulatory *E. coli* lipopolysaccharide (10 ng/mL) concentration was utilized alone or co-incubated with KSL-W before applying to a monolayer of cells in their third to fourth passage, as described in the text. After a 4-h incubation period, the plates were washed and assayed for the presence of E-selectin by an enzyme-linked immunosorbent assay utilizing a monoclonal antibody to E-selectin. The results indicate that a significant reduction ($p < 0.0001$) of lipopolysaccharide-induced E-selectin expression occurred when KSL-W was co-applied over a concentration range of 25–200 μg/mL. The data presented are representative of at least three separate experiments using triplicate wells for each sample. A_{450}, absorbance at 450 nm; LPS, lipopolysaccharide.

KSL-W inhibits lipopolysaccharide hemagglutination in red blood cells

The ability of lipopolysaccharide to induce erythrocyte hemagglutination has been reported as a functional property of the direct interaction of erythrocytes with differing segments within the polysaccharide portion (30,33,42) as well as with the lipid A segment (43) of the lipopolysaccharide molecule. Therefore, to explore whether KSL-W binding to lipopolysaccharide would alter the lipopolysaccharide-induced hemagglutination of rabbit erythrocytes, we compared various *E. coli* lipopolysaccharide sample concentrations pre-incubated with the KSL-W peptide with matched lipopolysaccharide control samples alone (Fig. 4). For experimental controls, *P. intermedia* strain #17 whole cells caused routine and consistent hemagglutination of washed rabbit erythrocytes (positive control), whereas phosphate-buffered saline alone did not induce any hemagglutination (Fig. 4B). The results from these experiments indicated that KSL-W, at a fixed concentration, was able to block the induced hemagglutination over a wide and increasing dose range of lipopolysaccharide (Fig. 4A). Blocking or overwhelming this observed hemagglutination inhibition was achieved at a lipopolysaccharide : peptide ratio of approximately 5:1 (Fig. 4B). Furthermore, the assay design revealed that the application of a constant amount of KSL-W (25 μg/well) did not spontaneously result in a rabbit erythrocyte hemagglutination reaction (see Fig. 4A, lipopolysaccharide 0 μg/well + KSL-W 25 μg/well co-incubation) and allowed for functional hemagglutination activity when lipopolysaccharide concentrations became maximal (see Fig. 4B, lipopolysaccharide 125 μg/well + KSL-W 25 μg/well).

KSL-W inhibits lipopolysaccharide from oral bacteria

It has been reported that structural variations exist between lipopolysaccharide samples that have been extracted from different gram-negative bacteria (44). Lipopolysaccharide structural variation has also been shown to be critical in the differential host responses towards different oral bacterial lipopolysaccharide samples (8,45,46). Therefore, because of the observed heterogeneity that exists within lipopolysaccharide between (and within) different genera, several different oral bacterial lipopolysaccharide samples were extracted, purified and co-incubated with the KSL-W peptide, and then screened in the recombinant Factor C endotoxin assay. These experimental assays revealed that KSL-W, at a starting concentration of 76.4 μM, was able to neutralize approximately 70–90% of the oral bacterial lipopolysaccharide tested (Fig. 5). However, a 10-fold decrease in this starting concentration resulted in a substantial reduction of lipopolysaccharide inhibition from the *F. nucleatum* 10953 and *P. gingivalis* 33277 samples. This variability in KSL-W-induced inhibition was evident in the 50% inhibition calculations for the different oral lipopolysaccharide samples tested (*P. gingivalis* 33277

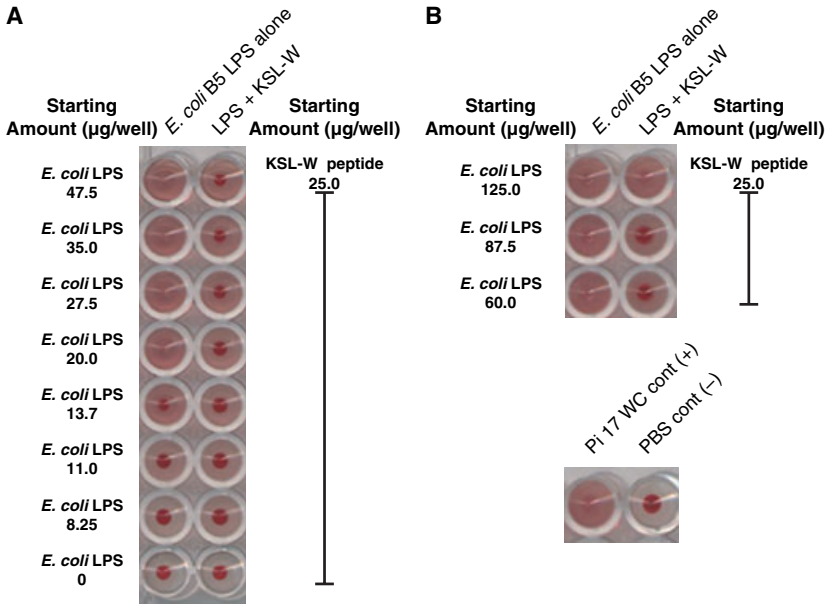

Fig. 4. Hemagglutination assay in rabbit erythrocytes. Equal volumes of a standard, fixed concentration of the KSL-W peptide (500 μg/mL) were co-incubated with increasing concentrations of *Escherichia coli* O55:B5 lipopolysaccharide resuspended in phosphate-buffered saline. The final results were scored visually and against experimental controls as described in the Material and methods. (A) The results indicate that hemagglutination was inhibited with the KSL-W–lipopolysaccharide co-incubated samples over a range of concentrations compared with the lipopolysaccharide-stimulated control lanes. (B) This observed hemagglutination inhibition by KSL-W was abrogated using a 5:1 ratio of *E. coli* O55:B5 lipopolysaccharide : KSL-W. Data displayed were taken from one of more than three separate and independent experiments and also served as a visual representation of results. LPS, lipopolysaccharide; Pi 17 WC, *Prevotella intermedia* 17 whole cells; PBS, phosphate-buffered saline.

lipopolysaccharide 50% inhibition = 17.2–51.8 μM; *P. intermedia* 25611 lipopolysaccharide 50% inhibition = 3.9–5.4 μM; *A. actinomycetemcomitans* 43718 50% inhibition = 5.1–6.7 μM; *F. nucleatum* 10953 50% inhibition = 9.4–45.0 μM).

Discussion

In this study, we investigated whether an antimicrobial decapeptide (KSL-W), was capable of binding to and neutralizing the effects of lipopolysaccharide. Bacterial components such as lipopolysaccharide are continuously released into locally infected tissue as a result of naturally occurring bacterial cell growth and lysis but are subsequently scavenged and cleared by host innate cells. However, it has been shown that antibiotic treatment of bacterial infections releases significant quantities of toxic products as a result of the systematic disruption and lysis of bacterial cells (47,48), which may, in turn, overwhelm the abilities of these local host cells to clear infected tissues. It is also known that release of toxic products, such as lipopolysaccharide, into the bloodstream can set up a septic state and if unregulated, this systemic lipopolysaccharide-induced cytokine release can lead to devastating organ system compromise or failure associated with septic shock (4,49,50).

Using ELISA-based techniques, we demonstrated that the KSL-W peptide was able to bind to *E. coli* O55:B5 lipopolysaccharide. Peptide-binding saturation was achieved with KSL-W at an approximate starting concentration of 500 μM. This was not observed in the KSL-W analog (A6), which was designed using the (positively charged) K → (neutral) V replacement scheme, suggesting that the KSL-W–lipopolysaccharide binding was related more to the cationic charge of the molecule than the hydrophobic residue interaction. However, we are still trying to ascertain the extent of the effects that these hydrophobic interactions may have regarding the stability of the binding event between the peptide and lipopolysaccharide (or the lipid A component).

Cationic peptide binding to lipopolysaccharide has been well documented and has been related to a number of structural features contained within the peptide itself (16–18,36,51–54). Although specific charge groups and overall length of the peptide have been shown to be essential in the lipopolysaccharide–peptide binding interaction (11,12,15,55), increasing evidence is mounting which suggests that even small or shortened peptides (< 15 mers) retain significant lipopolysaccharide binding and immunomodulatory function (13,15,56). Additional experimental analysis with cationic charge elimination or significant truncation of the KSL-W decapeptide [KSL-W analogs (A1–5)] resulted in variable (reduced or absent) binding to lipopolysaccharide within our assay systems (data not shown). This is in agreement with the results of Scott *et al.*, who, using the dansyl polymyxin B displacement assay, reported that the binding affinity to

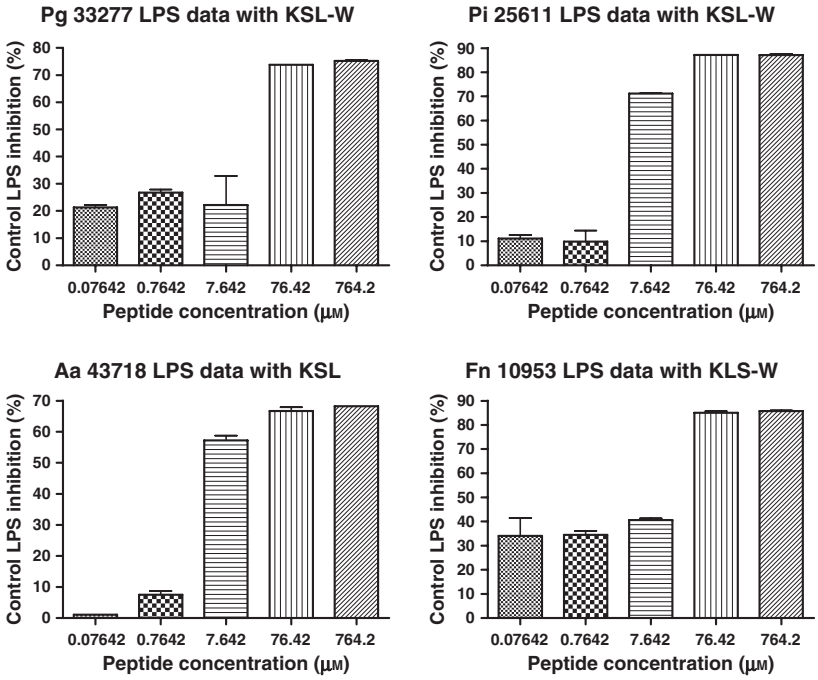

Fig. 5. Recombinant Factor C assay and neutralization of oral lipopolysaccharide. Different oral lipopolysaccharide samples from selected periodontopathic bacteria were extracted from lyophilized whole bacterial cells as described in the Material and methods. Different concentrations of the KSL-W peptide were incubated for 1 h at 37°C with equal volumes of a fixed and standardized concentration of oral lipopolysaccharide. Percentage inhibition of fluorescence was calculated as previously described. The results indicate that binding of KSL-W to oral lipopolysaccharide inhibits the lipopolysaccharide-induced recombinant Factor C fluorescence in a dose-dependent manner that was unique for each oral lipopolysaccharide sample tested. Mean results and standard deviation are displayed as a composite and a representative example obtained from three individual and independent assay results for each optimized oral lipopolysaccharide sample and assay control. Aa, *Aggregatibacter actinomycetemcomitans* 43718; Fn, *Fusobacterium nucleatum* 10953; LPS, lipopolysaccharide; Pg, *Porphyromonas gingivalis* 33277; Pi, *Prevotella intermedia* 25611.

E. coli O111:B4 lipopolysaccharide was considerably affected when alterations in charge, hydrophobicity or length were employed during synthesis of a similar type of α-helical peptide (36).

Interestingly, previous information regarding the biologic activity of cationic peptides was mainly focused on antimicrobial spectrums (13). However, recent reports have determined a wide range of biologic activity for both naturally occurring as well as synthetically derived peptides (11–14,16,17). In a commercial recombinant Factor C–lipopolysaccharide detection assay, a KSL-W peptide starting concentration of 7.6 μM was able to inhibit approximately 85% of the lipopolysaccharide present in the assay system. This observation is also in agreement with a recent study by Li *et al*. in which they demonstrated an approximate 80% inhibition of lipopolysaccharide using a 10 μM concentration of the Sushi 3 antimicrobial peptide (34 mer) within the same commercial assay system. Ten-fold concentration changes (increase/decrease) dramatically altered the ability of the Sushi 3 peptide to neutralize lipopolysaccharide, which was also observed in our experimental assay using KSL-W. We then extended this observation to include experimentation with oral lipopolysaccharide isolated from selected groups of periodontopathic organisms. Similar neutralization effects were also observed with lipopolysaccharide obtained from these oral bacteria samples. These results suggest that small, positively charged peptides such as KSL-W may potentially serve as a therapeutic adjunct to attenuate some of the toxic effects associated with bacterial-induced inflammatory periodontal disease(s).

Although multiple lipopolysaccharide effects have been reported to be obtunded by various types of antimicrobial peptides (16–18,36,52–54,57), little is known about the effects on E-selectin expression or hemagglutination. The present study also showed that the decapeptide KSL-W, throughout a wide concentration range (25–200 μg/mL) was able to obtund the lipopolysaccharide-induced expression of E-selectin in human umbilical vein endothelial cells. Similarly, a fixed KSL-W peptide concentration was able to block the lipopolysaccharide-induced hemagglutination of rabbit red blood cells over a wide lipopolysaccharide stimulatory range. Previous reports detailing the hemagglutination activities of lipopolysaccharide have sometimes been performed after specific portions of the lipopolysaccharide molecule have been removed or purified to define or enhance activity (42,43). Here we used commercially obtained lipopolysaccharide and rabbit erythrocytes to determine if this peptide could block

the inherent hemagglutination ability of a standard, unaltered commercial lipopolysaccharide. Further investigation of the extent of, as well as elucidation of the specific mechanisms responsible for this peptide-induced hemagglutination inhibition (utilizing different lipopolysaccharide samples within different types of erythrocytes), will need to occur.

It has been reported that toxicity towards mammalian cells is a significant concern regarding many antimicrobial peptides (1,58). To address this issue we incorporated the interleukin-1β induction of E-selectin (which stimulates E-selectin expression through a different extracellular component than lipopolysaccharide) as a positive control as well as a cellular vitality reporter in the human umbilical vein endothelial cells assay system. From this we were able to determine that within these cells, the interleukin-1β-induced E-selectin expression was similar to control values, even when co-applied with concentrations as high as 300–500 μg/mL of the KSL-W peptide. Additional Trypan blue experiments in human umbilical vein endothelial cells suggested that concentrations at or above these values were associated with mild-to-moderate cellular perturbation and therefore we limited our reported experimental observations to below these ranges. Furthermore, rabbit erythrocytes were functional, and agglutination ability was consistently unaltered in the presence of the KSL-W peptide at concentrations up to 250–500 μg/mL. This coincides with previous observations that KSL, the parent peptide of KSL-W, when applied at concentrations up to 1 mg/mL did not induce the cellular death of treated human gingival fibroblasts, as reflected by the ability of treated cells to reduce 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) at the same levels as the untreated cells (21). Using the same MTT assay, we also demonstrated that KSL-W at concentrations as high as 500 μg/mL did not induce the cell death of treated Vero cells (data not shown). Together, the results generated thus far have suggested that the concentrations reported in this study are nontoxic to mammalian cells.

In total, increasing evidence reveals that certain antimicrobial peptides do possess the ability to bind to and, either directly or indirectly, alter specific host responses towards lipopolysaccharide. Furthermore, smaller or truncated products of larger parent peptides have been shown to retain potent antimicrobial activity as well as specific and important immunomodulatory abilities. Smaller peptides have many unique advantages that permit simplified synthesis and alteration, which enables increased screening for these various modulatory properties. Further analysis will be required to determine the overall effect that smaller antimicrobial peptides, such as KSL-W, will have in relation to or combined with other innate host response elements, as well as being employed as potential therapeutic adjuncts.

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