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Lipopolysaccharide Structure Determines Ionic and Hydrophobic Binding of a Cationic Antimicrobial Neutrophil Granule Protein

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Bactericidal activity and binding of a 57,000-dalton cationic antimicrobial neutrophil granule protein (CAP57) are determined by the presence on bacteria of O-antigen polysaccharide chains and the availability of negatively charged groups in the lipid A region, the inner core region, or both regions of lipopolysaccharide. Polymyxin B (PMB)-resistant mutants with well-defined alterations in lipid A structure and charge (*pmrA*) are also more resistant to CAP57. We used biologically active radioiodinated CAP57 to study the characteristics and kinetics of binding to a sensitive Rb lipopolysaccharide chemotype, *Salmonella typhimurium* SH9178, and the relatively resistant *pmrA* mutant strain SH7426. Binding occurred rapidly and was specific and saturable. Because CAP57 appears to be bound in a manner similar to that of PMB, competition binding studies were performed. Excess PMB did compete with CAP57 for binding to SH9178. Nonapeptide, a polycationic derivative of PMB that has lost its hydrophobic portions, demonstrated a marked decrease in ability to compete for binding with CAP57 compared with PMB. This demonstrated the importance of hydrophobic binding in the interaction of CAP57 with the microbial surface. Thus, we have shown that binding of CAP57 to SH9178 is specific, saturable, and similar to binding of PMB. Both hydrophobic and ionic properties of CAP57 appear to be necessary for binding.

We have purified a 57,000-dalton cationic antimicrobial protein (CAP57) from human polymorphonuclear leukocyte granules that exhibits potent yet selective nonoxidative bactericidal action against certain gram-negative bacteria (6). Using highly purified CAP57, we have shown that lipopolysaccharide (LPS) structure determines phenotypic levels of resistance to the antimicrobial effects of CAP57 (2, 6) in that mutations that reduce the type and amount of sugars in LPS progressively decrease resistance to CAP57.

We have suggested that binding of CAP57 to the outer membrane of gram-negative bacteria, a prerequisite event for bactericidal activity, involves a three-step process. (i) Hydrophobic CAP57 must overcome hydrophilic repulsion due to the presence of sugar residues in the O-antigen and core region of LPS. (ii) Negatively charged residues within the lipid A region, the inner core region, or both regions of LPS may provide sites for initial ionic interaction of CAP57 with the microbial surface. Previous work has demonstrated clearly that the length of the hydrophilic carbohydrate in the O-antigen limits the antimicrobial activity of CAP57 (5), and we have shown recently an inverse correlation between O-antigen length and binding of CAP57 (2). (iii) Proposed step 3 involves interaction of CAP57 with the hydrophobic domains on lipid A that may serve to stabilize the binding of CAP57 to the microbial surface and thus facilitate its antimicrobial action. Weiss et al. have presented evidence to support ionic and hydrophobic antimicrobial interactions of a similar cationic neutrophil granule protein (13).

It has been found that polymyxin B (PMB)-resistant mutants of *Salmonella typhimurium* (*pmrA*) are also more resistant to CAP57 (6). The *pmrA* mutation is known to be associated with increased substitution of 4-aminoarabinose at the 4′ phosphate on lipid A and more ethanolamine-containing compounds within the LPS (9). These substitutions block anionic groups on lipid A which are potential binding sites for CAP57, add a positively charged amino group, and decrease the hydrophobicity of lipid A because of the presence of arabinose. This mechanism of increased resistance to CAP57 appears to be independent of O-side chain length and can occur even in the most sensitive deep-rough (Re LPS) strains.

We therefore designed experiments to ascertain whether CAP57 binds to the surface of *S. typhimurium* in a specific and saturable manner and to test the effects of the LPS O-side chain and the *pmrA* mutation on binding of CAP57 to the microbial surface. Using a direct assay for binding, we showed that CAP57 binds to *S. typhimurium* in a specific and saturable manner and found it to be similar to that of PMB (8). Moreover, the presence of O-antigen or 4-aminoarabinose attached to the 4′ phosphate of lipid A reduced the capacity of CAP57 to bind to the microbial cell surface.

MATERIALS AND METHODS

Bacterial strains and antimicrobial assays. Three isogenic strains of *S. typhimurium* were evaluated in these studies: LT2, a wild-type strain with fully smooth LPS; SH9178, an Rb LPS chemotype strain (*pmrA*$^+$); and SH7426, an Rb LPS chemotype, *pmrA* mutant strain (8).

Antimicrobial assays were performed as described by Rest et al. (4). Antimicrobial assays were performed in sterile microtiter trays with 10^3 bacteria in 0.1 ml of tryptone-saline per well. CAP57 (unlabeled or radiolabeled) in 0.1 ml of phosphate-buffered saline (pH 6.5) was added in appropriate concentrations, and incubation was allowed to proceed for 1 h at 37°C. Controls consisted of incubation of bacteria in the absence of CAP57. After incubation, aliquots were plated onto Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) for colony counts. Survival of bacteria was determined by the following equation: % survival = (bacteria incubated with CAP57)/(bacteria incubated alone) × 100. Survival data were calculated only if growth occurred in control wells.

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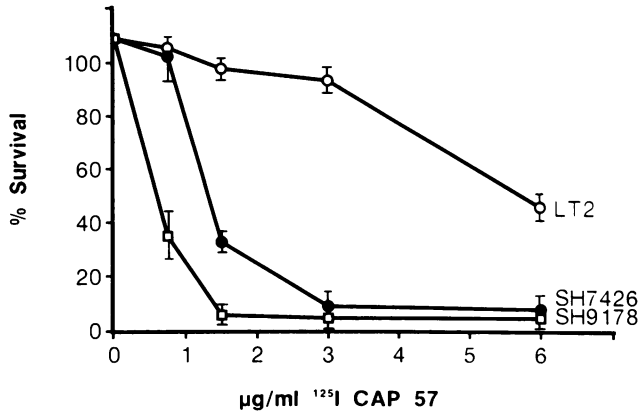

FIG. 1. Survival of *S. typhimurium* test strains after incubation with [^{125}I]CAP57 derived from human polymorphonuclear leukocyte granule extracts. Symbols: ○, sensitivity of strain LT2 (smooth LPS; LD_{50}, ca. 5.5 μg/ml); □, sensitivity of strain SH9178 (Rb LPS; *pmrA*$^+$; LD_{50}, ca. 0.5 μg/ml); ●, sensitivity of strain SH7426 (Rb LPS; *pmrA*; LD_{50}, ca. 1.2 μg/ml). Each datum point is the average of triplicate samples.

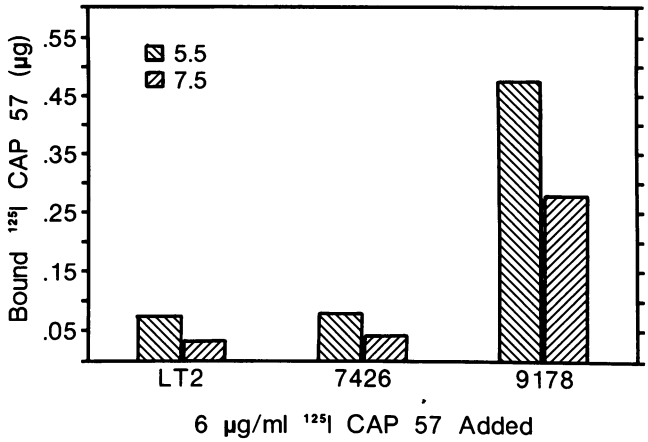

FIG. 2. Binding of [^{125}I]CAP57 to *S. typhimurium* at pH 5.5 and 7.5. Strains LT2, the wild-type smooth strain; SH7426 *pmrA*; and SH9178 *pmrA*$^+$ were incubated with 6 μg of [^{125}I]CAP57 per ml for 5 min at 37°C in tryptone-saline at pH 5.5 or 7.5.

Purification and iodination of CAP57. CAP57 was purified from human polymorphonuclear leukocyte granule extracts by sequential ion-exchange and molecular sieve chromatography as described by Shafer et al. (6) and modified by Casey et al. (1). Purified CAP57 was iodinated with ^{125}I by using a solid-phase Iodogen method (2). The iodination reaction was allowed to proceed for 10 min at 4°C in phosphate-buffered saline (pH 6.5), which resulted in an average specific activity of 10^5 cpm/μg of protein. The unreacted radioisotope was removed by overnight dialysis at 4°C.

Binding experiments. Binding of [^{125}I]CAP57 to test strains was determined as described previously (2). Appropriate amounts of radioiodinated CAP57 were introduced to 1.5-ml microcentrifuge tubes (Eppendorf) in a final volume of 0.1 ml. Bacterial suspensions in 0.1 ml of tryptone-saline (pH 5.5 or 7.5) containing approximately 10^7 CFU were added. Samples were incubated at 4 or 37°C for 5 to 30 min. After incubation, the bacteria were pelleted at ~10,000 × *g* (Beckman Microfuge) for 5 min and washed with 1 ml of tryptone-saline. Pellets were counted in a Beckman 5000 gamma counter.

Competition experiments were performed to determine specificity of binding. Increasing amounts of unlabeled CAP57 (1.25, 2.5, 5, 10, and 20 μg/ml) were added to a fixed amount of [^{125}I]CAP57 (2.0 μg/ml). In other competition experiments, excess PMB or nonapeptide (NP), a derivative of PMB which has lost its hydrophobic portion (10), was added to a fixed amount of [^{125}I]CAP57. The results were expressed as a percentage of the total CAP57 bound, with the amount of [^{125}I]CAP57 bound in the absence of competition designated as 100%.

RESULTS

Bactericidal activity of [^{125}I]CAP57. Figure 1 illustrates the altered resistance of the *pmrA* mutant of *S. typhimurium* SH7426 to [^{125}I]CAP57. At concentrations below 3 μg of [^{125}I]CAP57 per ml, there was significantly increased survival of the SH7426 (*pmrA*) strain compared with the parental strain SH9178 (*pmrA*$^+$). The fully smooth LT2 strain was consistently the strain most resistant to killing by [^{125}I]CAP57 (concentrations of [^{125}I]CAP57 resulting in 50% killing [LD_{50}] for SH9178, SH7426, and LT2 were <0.75, 1.2, and 5.5 μg/ml, respectively). The antimicrobial activity of [^{125}I]CAP57 as measured by LD_{50} was similar to that of unlabeled CAP57 (5). The bactericidal activity of CAP57 against all of the test strains was pH dependent; i.e., killing at pH 5.5 was greater than that at pH 6.5, which was greater than that at pH 7.5 (data not shown).

Binding. To correlate bactericidal activity with binding to bacteria, we further examined the *S. typhimurium* strains exposed to radiolabeled CAP57. Binding of [^{125}I]CAP57 to the test strains occurred rapidly, reaching near maximum within 5 min, with little additional protein bound by 30 min. All three test strains bound ~50% more CAP57 when incubation was performed at pH 5.5 than with incubation at pH 7.5 (Fig. 2). Raising the incubation temperature from 4 to 37°C had no effect on the binding of [^{125}I]CAP57 to strain LT2 or SH7426 but resulted in a 50% increase in binding to SH9178. Moreover, there was a marked reduction in total binding of CAP57 to SH7426 (*pmrA*) compared with the SH9178 parental strain (a three- to sixfold decrease based on multiple experiments). The reduced binding to SH7426 more closely resembled that of strain LT2 and correlated with its increased resistance to the antimicrobial effects of CAP57.

Binding characteristics. To determine binding specificity, we examined the effect of exposing the test strains to increasing amounts of unlabeled CAP57 on the binding of a fixed amount (2.0 μg/ml) of [^{125}I]CAP57 (Fig. 3). Incubation was performed at 4°C for 5 min. Unlabeled CAP57 competed very effectively for binding to SH9178 (*pmrA*$^+$). The competition was slightly less effective with SH7426 (*pmrA*), and there was little effective competition when binding to LT2 was evaluated. These data suggest that the proportion of specific binding of [^{125}I]CAP57 to SH9178 was greater than with the other test strains.

From the molecular weight (57,000) and the specific activity of [^{125}I]CAP57, we estimated that exposure of SH9178 to 20 μg of CAP57 per ml led to the binding of approximately 2×10^5 molecules per bacterial cell. Binding of CAP57 to the other strains was less: SH7426, 6×10^4 molecules per cell; LT2, 3×10^4 molecules per cell. The K_ms of binding, as calculated from the saturation curves, were 2.6×10^{-8} M for SH9178, 2.3×10^{-8} M for SH7426, and 7.4×10^{-8} M for LT2.

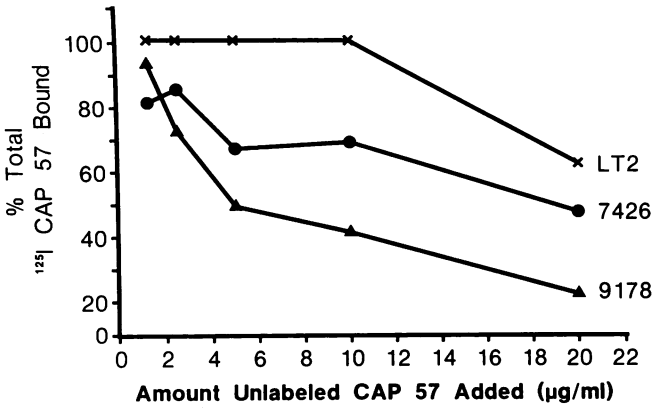

FIG. 3. Competition of unlabeled CAP57 and ^{125}I-labeled CAP57 for binding to *S. typhimurium* strains. Increasing amounts of unlabeled CAP57 were added to a fixed amount (2 μg/ml) of [^{125}I]CAP57. Results are expressed as the percentage of total CAP57 bound as measured in counts per minute; 100% is the amount bound in the absence of added unlabeled CAP57. Binding was measured after incubation at 4°C for 5 min.

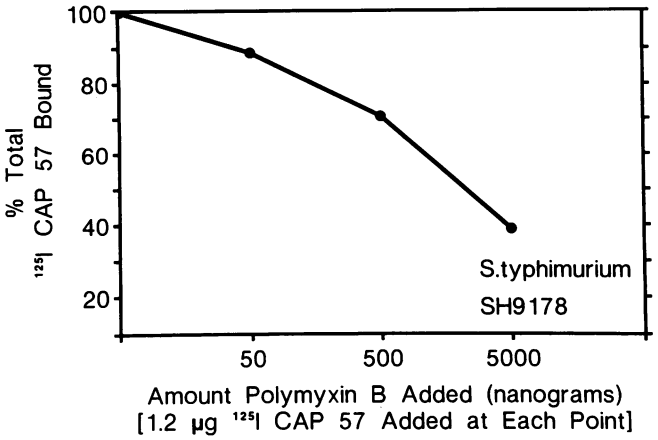

FIG. 5. Competition of PMB and ^{125}I-labeled CAP57 for binding to *S. typhimurium* SH9178. Increasing amounts of PMB were added simultaneously with a fixed amount (1.2 μg) of [^{125}I]CAP57. Results are expressed as the percentage of total [^{125}I]CAP57 bound in the absence of PMB. Binding was measured after incubation at 37°C for 5 min.

Figure 4 demonstrates saturable binding of [^{125}I]CAP57 to SH9178 and SH7426 when done at 4°C in the presence of increasing amounts of unlabeled CAP57. Saturation occurred after addition of similar amounts of labeled CAP57 for both strains, but the *pmrA* mutant bound a much lower quantity at saturation. In similar experiments, saturation was also demonstrated with the smooth LT2 strain but required addition of threefold higher protein concentrations (data not shown), suggesting that a substantial amount of CAP57 bound to this strain was due to nonspecific binding.

PMB or NP competition. Our finding that the *pmrA* mutation increased resistance to CAP57, apparently because of decreased binding capacity, suggested to us that *pmrA* mutants bound CAP57 in a manner similar to that of PMB. To test this hypothesis, we evaluated the ability of PMB to compete with labeled CAP57 for binding to strain SH9178. Binding experiments in which PMB and [^{125}I]CAP57 were added simultaneously and incubated at 37°C for 5 min demonstrated competition of PMB for binding at all concentrations tested (Fig. 5). A fourfold excess of PMB resulted in displacement of 68% of the [^{125}I]CAP57 bound in the absence of PMB.

To examine this competition further, we used a derivative of PMB called NP, which has had the fatty acid group and terminal diaminobutyric acid residue enzymatically removed. NP has previously been shown to bind to *S. typhimurium* but to exert markedly reduced antibacterial activity (11). There was a marked decrease in the ability of NP to compete for binding with CAP57 compared with PMB, suggesting less stable binding in the absence of the hydrophobic component, and binding of CAP57 to salmonella was mediated by ionic and hydrophobic bonds (Fig. 6).

DISCUSSION

By using radiolabeled CAP57, we demonstrated a direct correlation of protein binding to the microbial surface and its antimicrobial effects. The binding was relatively specific,

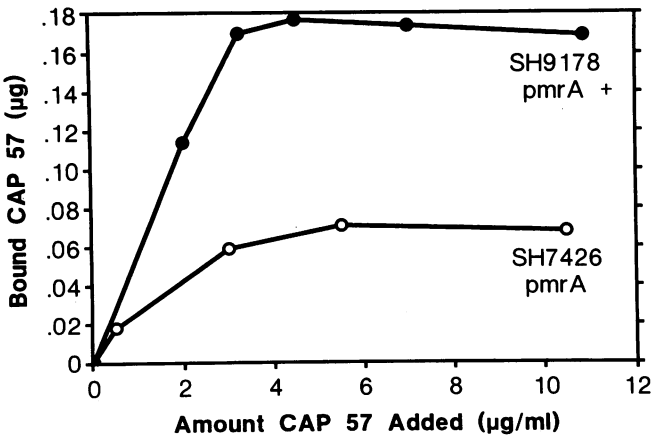

FIG. 4. Saturation binding of CAP57 to *S. typhimurium* SH9178 and SH7426. Increasing amounts of unlabeled CAP57 were added to a fixed amount (2 μg/ml) of ^{125}I-labeled CAP57. The specific activity of the radiolabel was adjusted for the total protein concentration. Binding was measured after incubation at 4°C for 5 min.

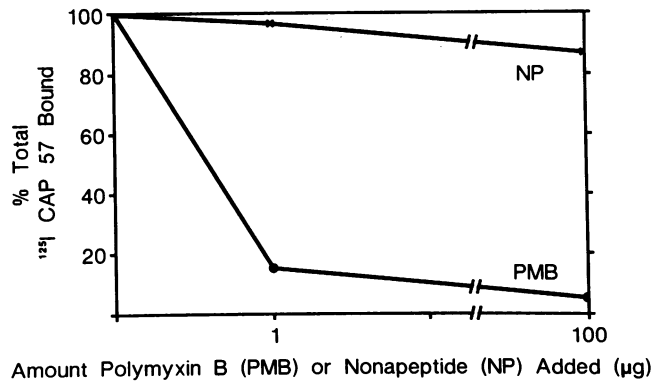

FIG. 6. Competition of PMB or NP with CAP57 for binding to *S. typhimurium* SH9178 (*pmrA*$^+$). Results are expressed as the percentage of total ^{125}I-labeled CAP57 bound to SH9178 when a fixed amount (0.1 μg) of CAP57 was added to various concentrations of PMB or NP; 100% represents the amount of [^{125}I]CAP57 bound when no PMB or NP was present. Incubation was at 37°C for 5 min.

saturable, and of high capacity. Further, we showed significant variation in specific binding capacity in salmonella strains with particular chemical-structural outer membrane alterations. Clearly, the strain most sensitive to the antimicrobial effects of CAP57 (SH9178) showed the highest proportion of specific binding.

Previous work has shown that sensitivity of bacteria to cationic granule proteins diminishes as the length of O-antigen and core oligosaccharide of LPS increases (4, 12). We have recently presented data that confirm the role of LPS O-antigen content in reducing total binding of CAP57 with bacteria (2). Externally projecting hydrophilic O-antigen material may limit access of the strongly cationic and hydrophobic CAP57 molecule to the negatively charged residues on lipid A. Hydrophilic repulsion of the highly hydrophobic CAP57 presumably reduces the number of molecules that interact with negatively charged residues located within the inner core region.

That the *pmrA* mutation known to affect the charge of the lipid A region, the inner core region, or both regions of LPS also decreased specific binding of CAP57 provides strong evidence that this region of LPS binds CAP57 via ionic interactions. The SH7426 (*pmrA*) strain was found to be more resistant to the antimicrobial effects of CAP57, bound fewer CAP57 molecules per cell, and had a lower proportion of specific binding when compared with the parental strain SH9178. The *pmrA* mutation also led to decreased hydrophobicity of lipid A as a result of arabinose substitution, thus lessening the potential for hydrophobic interactions with CAP57.

Strains LT2 and SH7426 are killed by CAP57, despite the differences in binding characteristics, but require higher protein concentrations to achieve killing comparable to that of SH9178. Binding of CAP57 to these less sensitive strains was not temperature dependent, which suggests a decreased influence of hydrophobicity on the process. Higher concentrations of CAP57 may therefore be required before adequate hydrophobic interaction is achieved and effective killing occurs.

The finding that PMB-resistant mutants (*pmrA*) of *S. typhimurium* were also more resistant to CAP57 presented an interesting corollary. PMB is a very hydrophobic and polycationic molecule that is thought to bind to the lipid A portion of LPS (3, 7). Morrison and Jacobs (3) showed convincingly that PMB binds to LPS via hydrophobic and ionic interactions. Competition of high concentrations of PMB with CAP57 for binding to SH9178 suggests not only the same binding sites but also similar binding characteristics with the microbial surface. Competition inhibition of CAP57 binding by PMB occurred with a K_i of 0.3 to 0.4 μg/ml. Our finding that the PMB derivative that has lost its hydrophobic portion but retained its polycationic properties (NP) was a very ineffective competitor for binding presented further valuable information. This lack of competition provides strong evidence that emphasizes the hydrophobic interactions of CAP57 with lipid A, which has previously been largely speculative. Further, the inability of polycationic NP to compete successfully with CAP57 suggests that altered hydrophobic binding to a large extent determines increased resistance of *pmrA* strains.

Taken together, our results support the following model for the binding of CAP57 to the surface of *S. typhimurium*. (i) Hydrophobic CAP57 must bypass the hydrophilic repulsion of the sugar residues in the core and O-antigen domains of LPS. (ii) The normally anionic region of lipid A is likely to play a role as a site for electrostatic interactions with the cationic CAP57 protein. (iii) Hydrophobic binding stabilizes the interaction and may result in membrane insertion of the protein. On the basis of our binding experiments, we suggest that both hydrophobic and ionic interactions of CAP57 with the lipid A region, the inner core region, or both regions of bacterial outer membranes are necessary for optimal antimicrobial binding of CAP57. The determination of other more complex factors that may influence interactions of CAP57 with the bacterial surface, such as LPS heterogeneity, will require further study.

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