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Antimicrobial Binding of a Radiolabeled Cationic Neutrophil Granule Protein

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A 57,000-dalton protein (CAP57) purified from human polymorphonuclear leukocytes has antimicrobial activity against a number of gram-negative bacteria. We developed a procedure using solid-phase Iodo-gen to radiolabel CAP57 without destroying its antibacterial activity. Iodinated and native CAP57 were electrophoretically identical. Autoradiographs of sodium dodecyl sulfate-polyacrylamide gels revealed >95% of the ^{125}I in a single heavy band in the 57,000-molecular-weight region. The quantity of [^{125}I]CAP57 bound to bacterial test strains was directly proportional to the sensitivity to CAP57.

Cationic proteins derived from the cytoplasmic granules of human polymorphonuclear leukocytes have been implicated as mediators in oxygen-independent killing of bacteria. A 57,000-dalton cationic antimicrobial protein (CAP57) has been purified from human polymorphonuclear leukocyte granules and found to kill a number of gram-negative organisms (2, 9). The structure of bacterial lipopolysaccharide (LPS) appears to partially determine levels of bacterial resistance to CAP57. Specifically, resistance is directly related to the length of the carbohydrates in the O antigen and core oligosaccharide. Lipid A, the anionic, hydrophobic head of the amphipathic LPS structure, may provide a binding site for CAP57 (9). Shafer et al. (8) have suggested that CAP57 binds to lipid A via ionic interactions with the 4′ phosphate residue.

Previous work with cationic antimicrobial proteins has suggested that binding of these proteins to the bacterial outer membrane is required for killing to occur (8, 11). However, direct evidence for the interaction of CAP57 with bacterial outer membranes has not been demonstrated. Accordingly, we sought to establish a procedure that would enable us to measure directly the binding of CAP57 to bacteria and to explore differences in surface binding to bacterial strains that vary in LPS composition. We report the capacity of extrinsically labeled CAP57 to bind to and kill *Salmonella typhimurium*.

Granulocyte concentrates (>95% polymorphonuclear leukocytes) obtained by leukapheresis from patients with chronic myelogenous leukemia were kindly provided by R. Vogler. The preparation and homogenization of polymorphonuclear leukocytes have been described (6). Granules, obtained by high-speed centrifugation, were extracted three times in 0.2 M sodium acetate (pH 4.0). CAP57 was prepared from the granule extracts by sequential ion-exchange and molecular-sieve chromatography as described by Shafer et al. (9) and as modified by Casey et al. (2). Before iodination the purified protein was dialyzed overnight at 4°C against phosphate-buffered saline (PBS) (pH 6.5). All protein concentrations were determined as described by Bradford (1).

The reaction vessel was prepared by application of 10 μg Iodo-gen (Pierce Chemical Co., Rockford, Ill.) dissolved in 100 μl of chloroform onto the surface of a glass tube (12 by 75 mm) and thorough drying under a stream of dry nitrogen gas at room temperature. The reaction vessel was rinsed with PBS buffer to remove microscopic flakes of Iodo-gen. Approximately 100 μg of CAP57 in a volume of 200 μl was added followed by 100 μCi of ^{125}I, and the reaction was allowed to proceed for 10 min at 4°C. The sample was then removed from the reaction vessel to terminate the iodination. Unlabeled NaI at a final concentration of 0.25 M was added to the iodination sample, and the volume was adjusted to a total of 1 ml. Unreacted ^{125}I was removed from the sample by overnight dialysis at 4°C.

Antimicrobial assays were performed as described by Rest et al. (7). ^{125}I-labeled CAP57 or unlabeled CAP57 was added to the wells of microtiter trays in appropriate amounts in a final volume of 0.1 ml of tryptone-saline (pH 7.0) (7). Bacterial suspensions (0.1 ml) containing ca. 1×10^3 to 5×10^3 CFU/ml were then added and incubated for 1 h at 37°C. Controls consisted of incubation of bacteria in the absence of CAP57. After incubation, 0.01-ml samples were plated onto Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) plates. The percent survival of bacteria was determined by the following equation: percent survival = $100 \times$ (number of bacteria incubated with CAP57/number of bacteria incubated alone). The following *S. typhimurium* strains were evaluated: LT2 (a wild-type strain with smooth LPS), SH9178 (Rb LPS), and SH7518 (Re LPS) (10).

Iodinated CAP57 was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was performed under reducing conditions with a 15% separating gel that contained a 4% stacking gel, as described by Laemmli (5). After electrophoresis, the gel was removed, washed overnight in 25% trichloroacetic acid, washed for 1 h in 10% acetic acid containing 1% glycerol, and dried. Autoradiography was performed by using X-Omat AR-5 film (Eastman Kodak Co., Rochester, N.Y.) with an intensifying screen. In some instances, the gel was stained with silver, as described by Hitchcock and Brown (4).

The reverse-phase high-performance liquid chromatography (RP-HPLC) elution profiles for both labeled and unlabeled CAP57 were determined. CAP57 in 0.2 M sodium acetate buffer was applied to a Waters C18 analytical RP-HPLC column (serial no. 096739; Waters Associates, Inc., Milford, Mass.). A linear gradient of 0.1% (vol/vol) trifluoroacetic acid (buffer A) and 60% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid (buffer B) was applied to the

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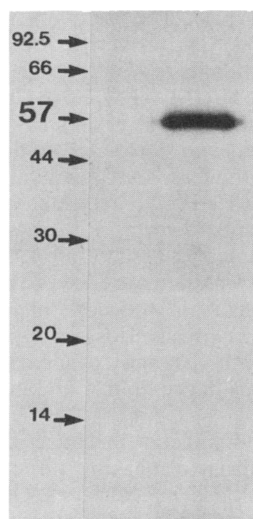

FIG. 1. SDS-PAGE analysis of iodinated CAP57. Shown is an autoradiograph of a dried 15% SDS-PAGE gel obtained with Kodak X-Omat AR-5 film exposed at −70°C by using an intensifying screen for 6 h. One microgram of [^{125}I]CAP57 was applied to the gel. The numbers in the margin indicate molecular masses in kilodaltons.

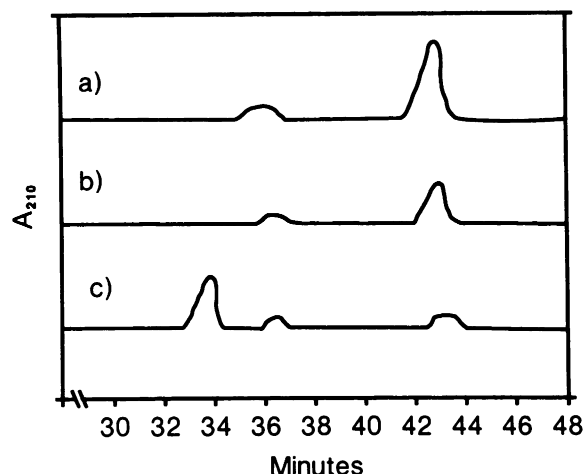

FIG. 2. RP-HPLC analysis of [^{125}I]CAP57. Protein was applied to a Waters C18 analytical RP-HPLC column. A linear gradient of 0.1% (vol/vol) trifluoroacetic acid and 60% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid was applied to the column at a flow rate of 1 ml/min. The A_{210} was determined. (a) Unlabeled CAP57 (20 μg) applied; (b) 5 μg of [^{125}I]CAP57 applied 2 days after iodination procedure; (c) 10 μg of [^{125}I]CAP57 applied 6 weeks after iodination procedure.

column at a flow rate of 1 ml/min. Fractions were collected at 1-min intervals, and the A_{210} was determined.

Appropriate amounts of radioiodinated CAP57 were introduced into microcentrifuge tubes, and the final volume was adjusted to 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 for 5 min and washed with 1 ml of tryptone-saline. After the supernatants were removed, the bacterial pellets were resuspended in tryptone-saline and transferred to a new vessel. The radioactivity in the supernatants and resuspended pellets was counted in a gamma counter (Beckman Instruments, Inc., Fullerton, Calif.). Selected samples were analyzed further. The resuspended bacterial pellets were solubilized in electrophoresis buffer and boiled for 3 min, and insoluble material was removed by centrifugation. The supernatant was then subjected to SDS-PAGE (as described above). After electrophoresis, the gels were dried and autoradiography was performed as described above.

CAP57 was trace labeled by the Iodo-gen method, with an average specific activity of 10^5 cpm/μg of protein. The typical incorporation of ^{125}I by CAP57 was 10 to 12%. Levels of protein-bound radioactivity, as determined by trichloroacetic acid precipitation, were consistently >90%. The preparation of [^{125}I]CAP57 was stored at 4°C in PBS (pH 6.5) and used within 7 days of iodination.

After iodination, the CAP57 preparation was analyzed by autoradiography of dried SDS-PAGE gels. Silver-stained gels containing unlabeled CAP57 and the iodinated product showed similar migration of both proteins (data not shown). Autoradiography of the dried SDS-PAGE gels revealed a heavy area of radioactivity coinciding with the 57,000-molecular-weight (57K) band (Fig. 1). The 57K band represented >95% of the radioactivity detected in the lane by density scanning.

The iodinated product was analyzed further by RP-HPLC. The [^{125}I]CAP57 preparation eluted identically to that of the unlabeled protein (Fig. 2a and b). However, when the iodinated preparation was reevaluated by RP-HPLC after 6 weeks of storage in PBS (pH 6.5) at 4°C, apparent degradation was noted (Fig. 2c). Accordingly, all further studies of binding and bactericidal activity were performed within 7 days of iodinating CAP57.

Bactericidal assays were performed with dilutions of unlabeled CAP57 or [^{125}I]CAP57. The percent survival of the *S. typhimurium* test strains exposed to various levels of [^{125}I]CAP57, ranging from 0.25 to 6 μg/ml, is shown in Fig. 3. The survival curves for both unlabeled (data not shown) and labeled CAP57 were similar. The sensitivity of the test strains to [^{125}I]CAP57 correlated with the LPS chemotype (Re > Rb > smooth) and was consistent with previous studies (8) using unlabeled CAP57.

Experiments were conducted to evaluate binding of [^{125}I]CAP57 to various strains of *S. typhimurium*. The bac-

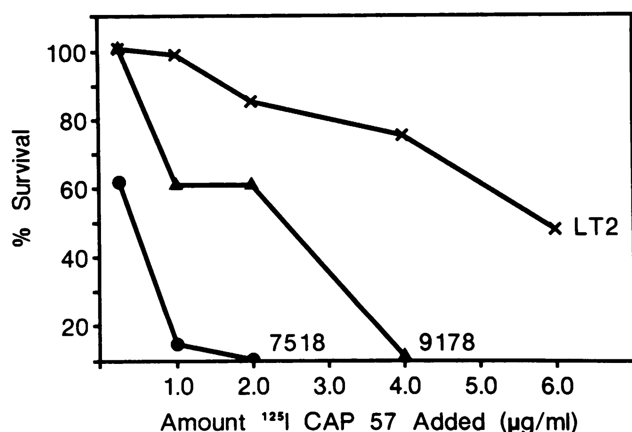

FIG. 3. Bactericidal activity of radiolabeled CAP57 against various *S. typhimurium* LPS chemotypes. Symbols: ×, strain LT2 (smooth LPS); ▲, strain SH9178 (Rb LPS); ●, strain SH7518 (Re LPS). Each datum point is the average of triplicate samples; values did not differ by more than 10%.

TABLE 1. Binding of [^{125}I]CAP57 to *S. typhimurium* strains

Strain	LPS chemotype	Amt of [^{125}I]CAP57 bound[a] at incubation concn (μg/ml):		
		1.25	2.5	5.0
SH7518	Re	0.077	0.153	0.275
SH9178	Rb	0.023	0.040	0.090
LT2	Smooth	0.013	0.024	0.041

[a] Amount of bound [^{125}I]CAP57 is expressed as micrograms per 10^7 cells and represents the average of a minimum of two separate assays. Background counts were subtracted before the protein equivalent was calculated. Incubation was at 4°C.

teria were exposed to CAP57 as described above. Repeated washing with PBS did not remove significant amounts of ^{125}I-labeled proteins or diminish background counts in the controls. The radioactivity in solubilized pellets was counted directly in a gamma counter after transfer to new vessels. Binding reached near maximal by 5 min of incubation, with little additional protein bound by 30 min. All strains tested demonstrated enhanced binding of [^{125}I]CAP57 at pH 5.5 as compared with that at pH 7.5. Raising the incubation temperature from 4 to 37°C had no effect on the binding of [^{125}I]CAP57 to strain LT2, but doubled the binding to the rough-LPS strains. The counts per minute in the bacterial pellets (Table 1) correlated with the levels of sensitivity of the strains to CAP57 (Fig. 3). Autoradiography of SDS-PAGE gels showing the radioactivity contained in the solubilized pellets of strains SH7518 and SH9178 revealed that the Re strain bound more CAP57 than did the Rb strain and that migration on SDS-polyacrylamide gels was unaltered after binding occurred (Fig. 4).

We describe successful iodination of a cationic antimicrobial protein derived from human granulocyte granules. This technique trace labels CAP57 while maintaining its biologic activity, electrophoretic mobility in SDS-polyacrylamide gels, and elution characteristics from an RP-HPLC C18 column. Previous attempts to radioiodinate a similar cationic antimicrobial protein have been unsuccessful due to loss of bactericidal activity (11). The Iodo-gen technique, a tyrosine-labeling method, has proven to be a gentle one that minimizes oxidation damage (3).

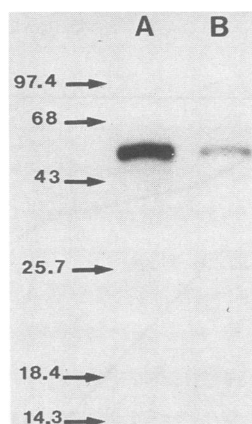

FIG. 4. Amounts of [^{125}I]CAP57 bound to two *S. typhimurium* strains. Pellets of bacteria with bound [^{125}I]CAP57 were solubilized in electrophoresis buffer (62.5 mM Tris hydrochloride [pH 6.8], 4% SDS, 10% β-mercaptoethanol, 10% glycerol, 0.1% bromophenol blue) and separated on a 15% SDS-polyacrylamide gel. After being dried, the gel was exposed to Kodak X-Omat AR-5 film for 12 h at −70°C. Lane A, Strain SH7518 (Re LPS); lane B, strain SH9178 (Rb LPS). The numbers in the margin indicate molecular masses in kilodaltons.

Using bactericidal [^{125}I]CAP57 in the binding assay, we found that levels of bacterial resistance to CAP57 among strains of *S. typhimurium* correlated with their capacity to bind CAP57. Thus, the less CAP57-resistant strain (SH7518; Re LPS) bound [^{125}I]CAP57 to a greater extent than did the more resistant strains bearing Rb or smooth LPS. We believe that the simplest explanation for the observation (8) that deep rough LPS mutants are less resistant than strains containing more sugars in the core oligosaccharide or O antigen is that the progressive loss of sugars in LPS due to mutations enhances the capacity of CAP57 to bind to negatively charged groups in the lipid A and inner core region of LPS.

Our results are significant because the binding assay described permitted us to measure directly the amount of CAP57 bound to bacterial cell surfaces. Others (11, 12) have used indirect assays to estimate the amount of a bactericidal protein (bactericidal/permeability-increasing protein). Although their studies suggest a role for LPS-cationic protein interactions, because of the nature of the assay system, defining parameters that distinguish specific from nonspecific binding is not feasible. The binding assay we describe permits critical evaluation of the bacterial cell surface structures that specifically bind CAP57, the affinity kinetics of binding, and the mechanisms (ionic or hydrophobic or both) of binding. Information gained should permit determination of the relation of bacterial binding of cationic antimicrobial proteins to bactericidal activity.

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