Lipopolysaccharide Masking of Gonococcal Outer-membrane Proteins Modulates Binding of Bactericidal Cathepsin G to Gonococci

By WILLIAM M. SHAFER

Department of Microbiology and Immunology, Emory University School of Medicine,
and The Veterans Administration, Atlanta, Ga. 30322, USA

(Received 22 June 1987; revised 13 October 1987)

Human polymorphonuclear leucocyte (PMN) lysosomal cathepsin G exerts potent bactericidal action against *Neisseria gonorrhoeae* *in vitro*, independent of its serine esterase activity. The results presented demonstrate that (1) bactericidal, diisopropylfluorophosphate-treated cathepsin G binds in a specific and saturable manner to the surface of gonococci, (2) loss of carbohydrates in gonococcal LPS due to mutation increases total and specific binding of cathepsin G, and (3) at least three outer-membrane proteins (OMPs) (PIA, PIII, and a 45 kDa OMP) interact with cathepsin G. Taken together, the results suggest that gonococcal susceptibility to the lethal action of cathepsin G, and perhaps susceptibility of gonococci to oxygen-independent killing by PMNs, is controlled by LPS-masking of cathepsin-G-binding OMPs.

INTRODUCTION

Human PMN lysosomal cathepsin G exerts potent bactericidal activity *in vitro* against *Neisseria gonorrhoeae* (Shafer *et al.*, 1986*a, b*). Hence, cathepsin G is a candidate for nonoxidative killing of gonococci by PMNs. This is important since previous studies (Casey *et al.*, 1986; Rest *et al.*, 1982) have demonstrated the capacity of PMNs to kill gonococci by nonoxidative means, in the absence of oxidative antimicrobial systems.

The antigonococcal action of cathepsin G is independent of its serine esterase activity (Shafer *et al.*, 1986*a, b*) and is probably due to its highly cationic nature (Odeberg & Olsson, 1975). Recent studies described a spontaneous LPS mutant of gonococci that exhibited hypersusceptibility to the lethal action of cathepsin G (Shafer *et al.*, 1986*a*). Using this mutant and its more cathepsin-G-resistant parental strain it was found that certain OMPs were candidates for binding cathepsin G since they were susceptible to cleavage by enzymically active cathepsin G (Shafer & Morse, 1987). Moreover, surface-exposure and susceptibility of OMPs PIII and PIA appeared to be greater in the OM of the LPS mutant. This suggested that the mutation affecting LPS structure augmented exposure of these OMPs due to loss of carbohydrates that normally mask them.

Enhanced susceptibility of OMPs in the LPS mutant to proteolytic cleavage by cathepsin G suggested that their greater surface exposure might promote binding of cathepsin G. To test this hypothesis, the binding of radiolabelled cathepsin G to the surface of isogenic strains differing in susceptibility to the lethal action of cathepsin G was evaluated.

METHODS

Preparation and iodination of cathepsin G. Human PMN lysosomal cathepsin G was prepared from acetate extracts (Rest *et al.*, 1977) of isolated granules as described by Shafer *et al.* (1986*b*). The granulocyte donors denied previous history of gonococcal infection and were not under antibiotic treatment. Using Iodogen (Pierce), 100 µg

Abbreviations: DFP, diisopropylfluorophosphate; OMP, outer-membrane protein; PMN, polymorphonuclear leucocyte.

purified cathepsin G, determined by the method of Bradford (1976), was iodinated as described by Farley *et al.* (1987). After iodination, samples were treated with DFP (Shafer *et al.*, 1984) and dialysed at 4 °C against several changes of PBS (0.145 M-sodium chloride, 0.145 M-sodium phosphate, pH 7.4). The specific activity of the radioiodinated cathepsin G (with or without DFP treatment) was in the range $1-5 \times 10^5$ c.p.m. per μg cathepsin G. Radioiodinated preparations of cathepsin G were examined by SDS-PAGE (Laemmli, 1970) under reducing conditions. The slab gel was stained with silver (Shafer *et al.*, 1984), dried onto Whatman filter paper and applied to Kodak X-OMAT AR-5 X-ray film for autoradiography. The results (Fig. 1) showed that the DFP-treated cathepsin G migrated as a 24–25.5 kDa triplet (lane A), characteristic of the known isoenzymes (Travis *et al.*, 1980), while the enzymically active preparation revealed the presence of minute quantities of additional polypeptides of molecular mass <14 kDa (lane B).

Strains of N. gonorrhoeae. Strains FA102 and WS1 were used throughout this study. Strain WS1, a spontaneous pyocin-resistant mutant of FA102, produces a truncated LPS (Shafer *et al.*, 1986a) but has a similar OMP profile to parental strain FA102 (Shafer & Morse, 1987); both strains produce PIA, serovar PIA1. WS1 is at least tenfold more susceptible than FA102 to the lethal action of enzymically active and inactive preparations of cathepsin G (Shafer *et al.*, 1986a, b). For routine culturing, both strains were passed on GCB agar containing defined supplements (Shafer *et al.*, 1986a) as OP⁻P⁻ variants.

Analysis of the binding of cathepsin G to gonococci. Gonococci were harvested from GCB agar plates and grown in GCB broth containing supplements and 0.043% (w/v) sodium bicarbonate. All cultures were grown at 37 °C with shaking to an OD₅₅₀ of 0.35. Samples (1 ml) were added to Eppendorf microfuge tubes containing iodinated cathepsin G, and after brief vortexing, the mixtures were incubated at the indicated temperature. At indicated intervals, samples were removed and the bacteria were collected by centrifugation for 2 min at 13 500 g. The supernatants were removed and the pellets analysed for bound ¹²⁵I using a Beckman LS 4000 gamma scintillation counter. Each experiment was done in triplicate. In the absence of added gonococci, about 10% of the labelled cathepsin G bound to the sides of the microfuge tubes when incubations were done in GCB broth alone under the same conditions. Binding was assessed as μg of cathepsin G bound per 10^7 c.f.u. added. In several experiments determination of the total number of diplococcal particles by Coulter counter (20 μm aperture) measurements showed that about 90% of the added gonococci (FA102 and WS1) were viable (viability was determined by plating on GCB agar).

Immunoprecipitation of gonococcal OMPs bound to cathepsin G. Surface-iodinated gonococci were obtained as described by Judd (1982). After washing with PBS, the labelled gonococci were added to microfuge tubes containing unlabelled cathepsin G (100 $\mu\text{g ml}^{-1}$) or an equivalent volume of PBS. The mixtures were incubated at 22 °C for 30 min, centrifuged, washed twice with PBS, and the pellet was resuspended in 100 μl 1% (w/v) SB-12 (Calbiochem, Behring Diagnostics) and incubated at 37 °C for 30 min. The SB-12 extracts were collected by centrifugation as described by Joiner *et al.* (1983), then treated with 500 μg IgG purified from a goat anti-cathepsin G antiserum (kindly provided by James Travis, University of Georgia, Athens, Ga.). After overnight incubation at 4 °C, the mixtures were treated with 25 μl rabbit anti-goat IgG antiserum (heavy and light chain specific; Cappel Labs) and incubated for 1 h at 37 °C. Immunoprecipitates were collected by centrifugation, treated with SDS-PAGE solubilizing buffer, boiled for 5 min and subjected to electrophoresis (Laemmli, 1970). After electrophoresis, the gel was stained with Coomassie Brilliant Blue, destained with 10% (v/v) acetic acid containing 1% (v/v) glycerol and dried onto filter paper. The dried gel was exposed to X-ray film at -80 °C for 24 h, the film developed, and the dried gel exposed to fresh X-ray film for an additional 96 h.

RESULTS

Earlier studies by Shafer & Morse (1987) showed that enzymically active cathepsin G cleaved gonococcal OMPs *in situ* while DFP-treated cathepsin G did not. Since both preparations of cathepsin G exerted similar bactericidal action (Shafer *et al.*, 1986a, b), experiments were designed to test whether DFP-treated cathepsin G binds to the surface of gonococci in a specific and saturable manner, whether loss of carbohydrates in LPS enhances binding and if surface-exposed OMPs interact with cathepsin G.

Binding of DFP-treated cathepsin G to strain FA102

Binding of DFP-treated cathepsin G, labelled with ¹²⁵I (Fig. 1, lane A), was first evaluated using strain FA102 in the standard binding assay described in Methods. The binding assay was done at 4, 22 and 37 °C with samples removed at various times. The results (not presented) showed that binding was rapid (complete within 1 min) and relatively independent of temperature. Approximately 18% of the added (1 μg) cathepsin G was bound to the gonococcal pellet.

Fig. 1

Fig. 2

Fig. 1. Autoradiographic profile of ^{125}I -labelled cathepsin G treated or not treated with DFP. Lane A, DFP-treated cathepsin G; lane B, untreated cathepsin G showing low-molecular-mass polypeptides resulting from autoproteolysis. The electrophoretic position of the molecular mass standards is shown.

Fig. 2. Specific and saturable binding of iodinated DFP-treated cathepsin G to *N. gonorrhoeae* strain FA102. ●—●, Amount of iodinated cathepsin G bound specifically, as calculated by total binding minus nonspecific binding; ●---●, percentage of the total cathepsin G bound specifically. See text for details.

Total and nonspecific binding of cathepsin G to whole cells of strain FA102 were distinguished by doing a binding experiment that employed increasing amounts of ^{125}I -labelled cathepsin G in the absence (total binding) or presence (nonspecific binding) of a 50-fold excess of unlabelled cathepsin G. Binding was evaluated after 5 min incubation at 22 °C because bactericidal activity of cathepsin G was not expressed under these incubation conditions (Shafer *et al.*, 1986b). Analysis of the calculated specific binding (equal to total minus nonspecific binding) revealed (Fig. 2) that binding was saturable. A binding constant of 10^8 M^{-1} was calculated when the data were graphed as a Scatchard plot (not shown).

Fig. 3. Loss of core oligosaccharides in LPS due to mutation results in increased total binding of iodinated cathepsin G to gonococci. Isogenic strains FA102 (●—●) and WS1 (●---●) were compared for differences in total binding of cathepsin G at 22 °C over a 30 min incubation period. The calculated number of molecules bound per viable diplococcus added is shown in the insert. ×—×, Ratio of number of molecules bound (WS1/FA102).

Table 1. *Specific binding of DFP-treated cathepsin G to N. gonorrhoeae FA102 and WS1*

Note that the incubation conditions used did not permit bactericidal activity. However, bactericidal action was evident when the incubation temperature was raised to 37 °C and samples were withdrawn after 15 but not 5 min.

Strain	¹²⁵ I-labelled cathepsin G bound	
	µg per 10 ⁷ c.f.u. added	Molecules per diplococcus
FA102	0.0156 ± 0.0028 (n = 3)*	3.83 × 10 ⁴
WS1	0.139 ± 0.0092 (n = 3)*	3.42 × 10 ⁵

* Means ± SD, with number of determinations in parentheses.

Gonococcal LPS structure modulates binding of cathepsin G

Isogenic strains FA102 and WS1, differing in LPS subunit molecular mass (Shafer *et al.*, 1986a) and susceptibility to cathepsin G (Shafer *et al.*, 1986a, b), were compared for their ability to bind DFP-treated cathepsin G. When incubated with 1 µg ¹²⁵I-labelled cathepsin G at 22 °C, strain WS1 exhibited enhanced total binding capabilities (Fig. 3); the number of molecules of cathepsin G bound per viable diplococcus added for each strain is presented in the table insert. Interestingly, binding of cathepsin G to strain WS1 increased with increasing time of incubation (Fig. 3).

Although the results from the binding experiment described above suggested that the mutation leading to truncation of LPS augmented binding of cathepsin G it did not address

Fig. 4. Detection of iodinated OMPs bound to cathepsin G in immunoprecipitates obtained from SB-12 extracts of strains FA102 and WS1. The surface-iodinated OMP profiles of strains WS1 (lane C) and FA102 (lane F) are shown for reference. The immunoprecipitates obtained from the SB-12 extracts of WS1 treated with cathepsin G (lane A) show a predominant PIA band and faint bands corresponding to PIII (●) and a 45 kDa OMP (▲). A much fainter PIA band is visible in the immunoprecipitate from strain WS1 not treated with cathepsin G (lane B). These OMPs were absent in the immunoprecipitates obtained from the SB-12 extracts of parental strain FA102 treated (lane D) or not treated (lane E) with DFP-treated cathepsin G.

whether this was due to increased specific binding. Accordingly, a binding experiment was done using ^{125}I -labelled cathepsin G in the presence and absence of excess unlabelled cathepsin G incubated with the test strains at 22 °C for 5 min. Consistent with the known differences in susceptibility to cathepsin G (Shafer *et al.*, 1986*a, b*), strain WS1 bound about eightfold more molecules of cathepsin G per added c.f.u. than strain FA102 (Table 1).

Enhanced binding of cathepsin G to OMPs due to spontaneous loss of carbohydrates in gonococcal LPS

Our earlier studies (Shafer & Morse, 1987) suggested that loss of sugars in the core region of the LPS from strain FA102 augmented the surface exposure of OMPs of strain WS1, thereby increasing their susceptibility to cleavage by cathepsin G. To test whether the OMPs of these strains differed in their ability to bind DFP-treated cathepsin G the strategy used by Joiner *et al.* (1983) in their work dealing with binding of C5b-9 to gonococcal OMPs was employed. In brief

this procedure is centred on the capacity of antiserum against bactericidal substances to coprecipitate ^{125}I -labelled OMPs from nonionic detergent extracts of bacteria treated with the appropriate antigen. Surface-iodinated suspensions of strains FA102 and WS1 were incubated in the presence or absence of DFP-treated cathepsin G ($100\text{ }\mu\text{g ml}^{-1}$) at $22\text{ }^{\circ}\text{C}$ for 30 min. After incubation, the bacteria were collected by centrifugation, washed three times with PBS and treated with 1% (w/v) SB-12 for 30 min at $37\text{ }^{\circ}\text{C}$. The debris was pelleted by centrifugation and the extract treated sequentially (see Methods) with goat anti-cathepsin G IgG and rabbit anti-goat IgG antiserum. The immunoprecipitates were washed, solubilized and subjected to SDS-PAGE. The iodinated gonococcal OMPs were detected by autoradiography of the dried gel. Significantly, the results (Fig. 4, lane A) clearly showed that PIA (serovar PIA1), was the predominant OMP coprecipitated with cathepsin G. Moreover, it was selectively coprecipitated with cathepsin G from SB-12 extracts obtained from suspensions of WS1 treated with cathepsin G. Two additional OMPs (PIII and a 45 kDa polypeptide) were also detected in the immunoprecipitates but in significantly smaller amounts than PIA. The OMP corresponding to PIII migrated identically to authentic PIII that was detected in immunoblots of whole-cell lysates using monoclonal antibody 2E6, known to recognize a surface-exposed epitope of PIII (Tam *et al.*, 1982). The immunoprecipitates from the SB-12 extracts obtained from the controls (absence of added cathepsin G) and cathepsin-G-treated FA102 (lane D) failed to reveal or showed substantially lower amounts of these OMPs (e.g. PIA in lane B but not lanes D and E). The results indicate that sugars present in the LPS of FA102 mask OMPs capable of binding cathepsin G. In control experiments (not shown) these OMPs were not precipitated when the anti-cathepsin G serum was omitted from the reaction mixture. Together these results indicate that sugars in the LPS of FA102 impede the binding of cathepsin G to the OMPs so that the absence of core oligosaccharides in the LPS of WS1 results in greater binding of cathepsin G to OMPs, thus permitting their detection.

DISCUSSION

The results from this study demonstrate that bactericidal yet enzymically inactive cathepsin G binds to the surface of gonococci in a saturable manner, indicating that binding is specific. This suggests that binding sites for cathepsin G exist on the surface of the gonococcal outer membrane. The finding that specific antibody against cathepsin G co-immunoprecipitated at least three OMPs (PIA, PIII and a 45 kDa protein) argues that enzymically inactive cathepsin binds to the surface of gonococci via interactions with OMPs. It remains to be determined whether these OMPs serve as primary binding sites for cathepsin G.

LPS may modulate the susceptibility of gonococci to the bactericidal action of cathepsin G by sterically reducing the binding of cathepsin G to the gonococcal cell surface. The use of isogenic strains differing in LPS subunit molecular mass has provided two indirect lines of evidence in support of the hypothesis that LPS may mask domains of surface-exposed OMPs that bind cathepsin G. Firstly, OMPs of the LPS mutant (WS1) appeared (Shafer & Morse, 1987) more susceptible to cleavage *in situ* by enzymically active cathepsin G than comparable OMPs of the parental strain. Secondly, in the experiments described in this report OMPs PIA1, PIII and a 45 kDa polypeptide co-immunoprecipitated with cathepsin G and could be detected only with extracts obtained from the mutant WS1. Failure to observe coprecipitation of OMPs, notably PIA, with extracts obtained from the parental strain FA102 was presumably due to the much reduced ability of this strain to bind cathepsin G. It is interesting to note that the 45 kDa OMP had a similar electrophoretic mobility to the recently described (Pohlner *et al.*, 1987) C-terminal portion of the pre-IgA protease which exists as an OMP after autoproteolytic cleavage, but additional work would be required to confirm this identity.

Work by others has shown that LPS associates with OMPs including PI (Hitchcock, 1984) and that PI is in turn complexed with PIII (McDade & Johnston, 1980; Newhall *et al.*, 1980). Thus, it was not surprising that these OMPs coprecipitated when the SB-12 extracts of cathepsin-G-treated gonococci were treated with antibody directed against cathepsin G. At present it is not clear whether this was due to binding of cathepsin G to LPS, PI or PIII, or a

combination of these surface components. Mutants lacking or having altered surface domains of these OMPs will help in distinguishing between these possibilities.

Detailing the molecular events leading to the binding of cathepsin G to the surface of gonococci and how this leads to bacterial death is clearly relevant since nonoxidative killing systems may be largely responsible for intraphagosomal death of gonococci. By understanding these events it may be possible to understand why some gonococci have appeared (Casey *et al.*, 1986) to persist within PMNs.

I thank J. Travis for providing the anti-cathepsin G IgG, R. Vogler for procuring granulocyte concentrates, V. Onunka and L. Martin for technical assistance, K. Wilkinson for suggestions regarding the binding assays, H. K. Ziegler and D. Stephens for critical review of the manuscript and the encouragement of J. K. Spitznagel. This work was supported by PHS grants AI 21150 (W. M. S.), AI 17662 (J. K. Spitznagel) and AI 22148 (W. Shafer and S. A. Morse) and the Veterans Administration Research Service.

REFERENCES

- BRADFORD, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.
- CASEY, S. G., SHAFER, W. M. & SPITZNAGEL, J. K. (1986). *Neisseria gonorrhoeae* survive intraleukocytic oxygen-independent antimicrobial capacities of anaerobic and aerobic granulocytes in the presence of pyocin lethal for extracellular gonococci. *Infection and Immunity* **52**, 384–389.
- FARLEY, M. F., SHAFER, W. M. & SPITZNAGEL, J. K. (1987). Antimicrobial binding of a radiolabeled cationic neutrophil granule protein. *Infection and Immunity* **55**, 1536–1539.
- HITCHCOCK, P. J. (1984). Analyses of gonococcal lipopolysaccharide in whole cell lysates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis: stable association of lipopolysaccharide with the major outer membrane protein (protein I) of *Neisseria gonorrhoeae*. *Infection and Immunity* **46**, 202–212.
- JOINER, K. A., WARREN, K. A., BROWN, E., SWANSON, J. & FRANK, M. M. (1983). Studies on the mechanism of bacterial resistance to complement-mediated killing. IV. C5b-9 forms high molecular weight complexes with bacterial outer membrane constituents on serum-resistant but not on serum-sensitive *Neisseria gonorrhoeae*. *Journal of Immunology* **131**, 1443–1451.
- JUDD, R. C. (1982). Surface peptide mapping of protein I and protein III of four strains of *Neisseria gonorrhoeae*. *Infection and Immunity* **37**, 632–636.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- MCDADE, R. L. & JOHNSTON, K. H. (1980). Characterization of serologically dominant outer membrane proteins of *Neisseria gonorrhoeae*. *Journal of Bacteriology* **141**, 1183–1191.
- NEWHALL, W. J., SAWYER, W. D. & HAAK, R. A. (1980). Crosslinking analysis of the outer membrane proteins of *Neisseria gonorrhoeae*. *Infection and Immunity* **28**, 785–792.
- ODEBERG, H. & OLSSON, I. (1975). Antibacterial activity of cationic proteins from human granulocytes. *Journal of Clinical Investigation* **56**, 1118–1124.
- POHLNER, J., HALTER, R., BEYREUTHER, K. & MEYER, T. F. (1987). Gene structure and extracellular secretion of *Neisseria gonorrhoeae* IgA protease. *Nature, London* **325**, 458–462.
- REST, R. F., COONEY, M. H. & SPITZNAGEL, J. K. (1977). Susceptibility of lipopolysaccharide mutants to the bactericidal action of human neutrophil lysosomal fractions. *Infection and Immunity* **16**, 145–151.
- REST, R. F., FISCHER, S. H., INGHAM, Z. Z. & JONES, J. F. (1982). Interactions of *Neisseria gonorrhoeae* with human neutrophils: effect of serum and gonococcal opacity on phagocytic killing and chemiluminescence. *Infection and Immunity* **36**, 737–742.
- SHAFER, W. M. & MORSE, S. A. (1987). Cleavage of the protein III and major iron-regulated protein of *Neisseria gonorrhoeae* by lysosomal cathepsin G. *Journal of General Microbiology* **133**, 155–162.
- SHAFER, W. M., MARTIN, L. E. & SPITZNAGEL, J. K. (1984). Cationic proteins isolated from human neutrophil granulocytes in the presence of diisopropyl fluorophosphate. *Infection and Immunity* **45**, 29–35.
- SHAFER, W. M., ONUNKA, V. C. & HITCHCOCK, P. J. (1986a). A spontaneous mutant of *Neisseria gonorrhoeae* with decreased resistance to neutrophil granule proteins. *Journal of Infectious Diseases* **153**, 410–417.
- SHAFER, W. M., ONUNKA, V. C. & MARTIN, L. E. (1986b). Antigonococcal activity of human neutrophil cathepsin G. *Infection and Immunity* **54**, 184–188.
- TAM, M. R., BUCHANAN, T. M., SANDSTROM, E. G., HOLMES, K. K., KNAPP, J. S., SIADAK, A. W. & NOWINSKI, R. C. (1982). Serological classification of *Neisseria gonorrhoeae* with monoclonal antibodies. *Infection and Immunity* **36**, 1042–1053.
- TRAVIS, T. J., GILES, P. J., PORCELLI, I., REILY, C. F., BAUGH, R. & POWERS, J. (1980). Human leukocyte elastase and cathepsin G: structural and functional characteristics. *CIBA Foundation Symposium: Excerpta Medica* **75**, 51–68.