Molecular Aspects of Complement-mediated Bacterial Killing

PERIPLASMIC CONVERSION OF C9 FROM A PROTOXIN TO A TOXIN*

Yunxia Wang‡, Edward S. Bjes and Alfred F. Esser§

From the Division of Cell Biology and Biophysics, School of Biological Sciences, University of Missouri-Kansas City, Kansas City, Missouri 64110

(Received for publication, November 9, 1999)

As part of the membrane attack complex complement protein C9 is responsible for direct killing of bacteria. Here we show that in the periplasmic space of an Escherichia coli cell C9 is converted from a protoxin to a toxin by periplasmic conditions missing in spheroplasts. This conversion is independent of the pathway by which C9 enters the periplasm. Both, C9 shocked into the periplasm and plasmid-expressed C9 targeted to the periplasm via a signal sequence are toxic. Toxicity requires disulfide-linked C9 because export into the periplasm of cells defective in disulfide bond synthesis (dsbA and dsbB mutants) is not toxic unless N-acetylcysteine is added externally to promote cystines. A N-terminal fragment, C9[1–144], is not toxic nor is cytoplasmically expressed C9, even in trxB mutants that are able to form disulfide bonds in the cytoplasm. Importantly, expression of full-length C9 in complement-resistant cells has no effect on their viability. Expression and translocation into the periplasm may provide a novel model to identify molecular mechanisms of other bacterial disulfide-linked proteins and to investigate the nature of bacterial complement resistance.

Complement is part of the innate immune system and one of the first lines of defense against pathogenic microorganisms. More than 20 blood complement proteins control microbial invasion of the host by two different mechanisms: (i) C3-mediated opsonization of cells and (ii) direct killing as a result of C5b-9 complex formation on the target (1). Much is known about the strategies used by pathogens to avoid opsonization because the basic molecular details of the process are recognized (1–3). In contrast, the mechanisms of direct complement-mediated killing of Gram-negative bacteria have remained elusive, and the means used by bacteria to become resistant to its effects are largely unknown (4). Without doubt, opsonization is of great importance in many infectious diseases. However, epidemiological studies of complement-resistant bacteria in systemic infections, as well as studies on individuals with genetically determined complement deficiencies, have indicated that (C5b-9)-mediated mechanisms play an important role in the control of Gram-negative infections. Most striking is the observation by Lassiter et al. (5) that Escherichia coli strains that are normally killed by adult serum (“serum-sensitive” strains) pose a severe threat to survival of premature or newborn infants because of diminished serum C9 concentrations in neonates.

Microbes escape complement destruction by inhibiting the three pathways of activation by synthesizing surface proteins that mimic complement control proteins or by capturing such proteins (1, 3, 4). Resistance to the action of C5b-9, also referred to as the membrane attack complex (MAC), is frequently achieved by changes from a rough to a smooth phenotype. Extension of surface carbohydrate structures causes an increase in the envelope hydrophobicity and weakens the anchoring of the MAC. Some strains escape killing by shedding of the MAC. Nevertheless, it is also known that not all smooth strains are serum-resistant and that some resistant strains do not shed the MAC but carry it in a stably bound form. Thus, strains that are truly resistant to complement, in that they are able to replicate in 50% serum (4), have acquired virulence factors in addition to those that change the chemical nature of the OM.

Some molecular details of the processes by which the MAC, once assembled on a bacterium, elicits death are currently understood. Death of sensitive strains following exposure to serum is extremely rapid, and it is thought to occur by dissipation of cellular energy (4, 6, 7). Killing can be prevented by incubation with membrane potential uncouplers or with inhibitors of oxidative phosphorylation indicating that ATP generated by the target bacterial cell is required. Efficient killing requires stable deposition of the C5b-9 complex on the OM but no other serum components in addition to the terminal complement proteins. Furthermore, it has been shown that formation of poly(C9), a tubular polymerization product of C9, is only incidental to hemolysis and not required for bacterial killing (7–9). However, whereas the (C5b-8),C9, complex is sufficient to lyse erythrocytes fully, it has no bactericidal activity (10).

This observation has been extended to show that a (C5b-8),C9, complex is strongly bactericidal but that incorporation of additional C9 molecules to form poly(C9) does not improve killing significantly (11). A further important conceptual advance was provided by Tomlinson et al. (12), who demonstrated that transfer of preassembled C5b-9 complexes into the OM by fusion techniques did not elicit cell killing despite the fact that small molecules now had access to the periplasm. Finally, Dankert and Esser (7) showed that the C-terminal half of C9, the C9b fragment, dissipated the membrane potential across respiring inner membrane (IM) vesicles, although the complete molecule had no effect. In addition, the requirement for a C9 receptor (that is, C5b-8 assembly) on the OM could be bypassed.

* This work was supported by University of Missouri Research Board Grant 1598, by National Institutes of Health Grants AI19478 and GM53748, and by a Marion Merrell Dow Professorship Endowment (to A. F. E.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† Present address: Dept. of Cell Biology, Neurobiology, and Anatomy, Medical College of Wisconsin, Milwaukee, WI 53226.
‡ Present address: Department of Biochemistry, Temple University, Philadelphia, PA 19140.
§ To whom correspondence should be addressed. Tel.: 816-235-5316; Fax: 816-235-1503; E-mail: essera@umkc.edu.

1 The abbreviations used are: MAC, membrane attack complex; C9*, C9 cleaved with a-thrombin; IM, inner membrane; OM, outer membrane; NaC, N-acetylcysteine; PAGE, polyacrylamide gel electrophoresis.
if the C9 molecule was osmotically shocked into the periplasmic space (13). This report firmly established that the bactericidal activity of complement is dependent upon C9 because none of the other terminal proteins when shocked into the periplasm elicited any cytotoxic effects. What is currently not understood is how C9, after its binding to the C5b-8 complex on the OM, translocates across the periplasm and dissipates the potential across the IM, whether bacterial envelope proteins are needed for cytotoxicity, or how C9, after its binding to the C5b-8 complex on the OM, translocates across the periplasm and dissipates the potential across the IM, whether bacterial envelope proteins are needed for cytotoxicity.

### EXPERIMENTAL PROCEDURES

**Complement Proteins and Assays**—Complement proteins C8 and C9 were purified and assayed for hemolytic activity according to standard procedures (14), and α-thrombin-cleaved C9 (C9b) was prepared as described (17). The spheroplasts were then incubated at 37 °C with trypsin (1 μg/ml) for up to 60 min, aliquots were withdrawn, and proteolysis was stopped by addition of SBTI (5 μg/ml). The cells were homogenized by sonication and analyzed by SDS-PAGE and ligand blotting.

**Ligand Blotting**—Published procedures were used to prepare total cell lysates and periplasmic shock fluids (25, 26) for SDS-PAGE analysis and blotting. A polyclonal anti-C9 serum adsorbed on immobilized cell extracts of *E. coli* BL21(DE3) or a monoclonal antibody (mAb216) against human C9 was used to detect recombinant C9 by Western blotting as described previously (23). Recombinant proteins were visualized using the nickel-nitrotriacetic acid alkaline phosphatase conjugate detection system (Qiagen, Valencia, CA).

**Proteolysis of Spheroplasts**—NLM100 cells were induced for 3 h to express secretable C9 (pYW60) or cytoplasmic C9 (pYW61), collected by centrifugation and resuspended in Tris-EDTA-sucrose buffer and lysed by three cycles of freeze-thawing and sonication, and total lysates were centrifuged for 16 h in a metrizamide gradient (1.27–1.29 g/ml) at 80,000 rpm in a Beckman TLA120.2 rotor to separate protein aggregates and membranes. Gradient fractions were analyzed by SDS-PAGE and ligand blotting.

**RESULTS**

**Effect of C9 on Viability of *E. coli* Spheroplasts**—We previously discovered that when C9 was shocked into the periplasm it killed sensitive *E. coli*, whereas it had no effect on respiring IM vesicles in contrast to the C9b fragment (7, 13). This prompted us to ask whether periplasmic processing was required for cytotoxicity. As shown in Fig. 1, neither native C9 nor C9 cleaved with thrombin (to produce C9a and C9b) had a significant effect on spheroplast viability. This strongly suggested that periplasmic factors are required for toxicity and indicated that proteolytic cleavage alone is inadequate to convert C9 from a protein toxin to a lethal factor.

**Expression of Recombinant C9 and Export into the Periplasm**—To gain further information on required periplasmic factors, we introduced C9 into the periplasm from the inside of the cell by secretion of recombinant C9, rather than from the outside by osmotic shock. The gene for mature C9 was cloned into the PET12b vector, which provides an OmpT signal peptide orientation to generate pYW54. Because these pET12b-derived plasmids could not be used for protein expression (see below) various complete C9 genes together with ribosomal binding sites and signal sequences (if any) were transferred as XbaI–S sacI fragments into PET22b and PASK75, respectively, to provide better control of transcription. The fragments were then inserted into the pET22b and pompA signals of the secretion vectors, and neither the intrinsic hexahistidine tag in pET22b nor the Strep Tag in pASK75 were fused to the C9 constructs. All nucleotide changes were verified by sequencing.

### TABLE I

| E. coli strain | Relevant marker | Source (Ref.) | Sensitivity (Ref.) |
|---------------|----------------|---------------|--------------------|
| C600          | Laboratory stock (13) | S (13)         |                    |
| LP1395        | Laboratory stock (13) | R (13)         |                    |
| ATCC 25922    | ATCC            | R (16)        |                    |
| BL21(DE3)     | dsbA::kan       | Novagen       | S (17)             |
| FD 596        | dsbB::Tn10-kan  | F. Dalley (15)| S (17)             |
| JCB773        | dsbA::kan       | J. Beckwith (16)| S (17)         |
| JCB792        | dsbB::kan       | J. Beckwith (16)| S (17)         |
| JCB775        | dsbA::kan, dsbB::kan | J. Beckwith (16)| S (17)         |
| YW112         | JCB775 (DE3)    | J. Beckwith (17)| S (17)         |
| GJ73          | dsbA::kan       | J. Beckwith (17)| S (17)         |
| NLM100        | dsbB C41Y      | J. Beckwith (17)| S (17)         |
| YW113         | NLM100(DE3)    | J. Beckwith (18)| S (17)         |
| AD49r         | trxB::kan       | J. Beckwith (18)| S (17)         |
| FC1           | BL21(DE3)::trxB::kan | F. Baneyx (19)| S (17)         |
| K17A1         | tolA::Tn10      | E. M. Click (20)| S (17)         |
| KP1032        | tonB::kan       | K. Postle (21)| S (17)             |

* S, complement-sensitive; R, complement-resistant.

* This work.
for export of recombinant proteins into the periplasm to generate pYW52. However, because of promoter leakage it was impossible to transform cells efficiently. When very large amounts of pYW52 DNA were used for transformation, a few colonies could be recovered. Sequencing of the plasmid isolated from four different colonies indicated that in each case the C9 gene was inactivated by insertion of bacterial IS elements (data not shown). Using vectors derived from pET22b or pASK75 that are more tightly controlled (pYW55-pYW61), transformants could be cultured. Expression of C9 caused an immediate loss of viability of the cells harboring these plasmids (Fig. 2, A and B). A secreted, N-terminal fragment, C9[1–144], was toxic, but the C-terminal fragment C9[145–538] was as toxic as the complete molecule (Fig. 2C). Significantly, when C9 was cloned into either vector without any signal peptide (pYW53, pYW54, pYW55, and pYW61), no toxic effects were observed after induction, and C9 accumulated as soluble and insoluble forms in the cytoplasm that were hemolytically inactive (data not shown). Of significance is the fact that expression of secretable C9 is a heavily disulfide-linked protein (Fig. 3A), which is inactive when reduced. Disulfide bond formation in the periplasm of E. coli is controlled by the dsbA, dsbB, dsbC, dsbD, dsbE, and dsbG genes (28). The DsbA protein is a protein disulfide isomerase in the periplasm that introduces cysteines into secreted proteins and thereby becomes reduced. The DsbB protein is an IM protein that reoxidizes reduced DsbA. In contrast to wild type cells, E. coli mutants with defective dsbA or dsbB genes harboring pASK75-derived plasmids were not

| Plasmid name | Parent plasmid/signal sequence | Insert | Tag | N-terminal sequence<sup>a</sup> | C-terminal sequence<sup>b</sup> |
|--------------|--------------------------------|--------|-----|----------------------------------|---------------------------------|
| pYW49        | pET12b/ompT                     | C9[1–144] |     | -STTS...                         | ...DPAANKARKEAEAAAAAAAAATG      |
| pYW50        | pET12b/ompT                     | C9[145–538] | His | DPLS...                           | ...LEFPNEK                      |
| pYW51        | pET12b/ompT                     | C9[145–538] | His | DPLS...                           | ...LEFPNEK                      |
| pYW52        | pET12b/ompT                     | mature C9 |     | -STTS...                         | ...DPNNSVKLAAEEHHHHHHH          |
| pYW53        | pET12b/none                     | C9[1–144] |     | MQYTTS...                         | ...DPAANKARKEAEAAAAAAAAATG      |
| pYW54        | pET12b/none                     | mature C9 |     | MQYTTS...                         | ...DPAANKARKEAEAAAAAAAAATG      |
| pYW55        | pET22b/ompT                     | C9[1–144] | His | -STTS...                         | ...DPNNSVKLAAEEHHHHHHH          |
| pYW56        | pET22b/ompT                     | C9[145–538] | His | -STTS...                         | ...DPNNSVKLAAEEHHHHHHH          |
| pYW57        | pET22b/ompT                     | mature C9 |     | -STTS...                         | ...DDPNNSVKLAAEEHHHHHHH         |
| pYW58        | pET22b/none                     | mature C9 |     | MQYTTS...                         | ...DDPNNSVKLAAEEHHHHHHH         |
| pYW59        | pASK75/ompT                     | C9[145–538] | His | -STTS...                         | ...DDPNNSVKLAAEEHHHHHHH         |
| pYW60        | pASK75/ompT                     | mature C9 |     | -STTS...                         | ...DDPNNSVKLAAEEHHHHHHH         |
| pYW61        | pASK75/none                     | mature C9 |     | MQYTTS...                         | ...DDPNNSVKLAAEEHHHHHHH         |
| pDNAKJ       | A. Gatenby (Du Pont)            |        |     |        | Cytoplasmic expression of Dnak and DnaJ |

<sup>a</sup> The parent plasmids pET12b and pET22b are from Novagen; pASK75 is from Biometra (Tampa, FL).

<sup>b</sup> Sequence differences from mature human C9 are indicated in bold italics.

**Fig. 1. Effect of C9 and C9<sup>a</sup> on viability of spheroplasts.** Viable C600 spheroplasts (1 × 10<sup>6</sup>ml) were incubated with 25 μg/ml purified C9<sup>a</sup> (lane a) or C9 (lane b) or bovine serum albumin (lane c) for 30 min at 37 °C, and viability was assayed by counting colonies grown overnight at 37 °C on agar plates prepared with LB medium.

**Fig. 2. Growth curves of E. coli expressing C9 and C9 fragments.** A and B, E. coli BL21(DE3) transformed with pYW60 (●) (A) or pYW57 (●) (B) and pYW58 (△) (B) were induced (dashed lines) to express periplasmic C9 (closed symbols) or cytoplasmic C9 (open symbols). C, expression of N-terminal C9[1–144] from pYW55 (▽) and C-terminal C9[145–538] from pYW56 (▲). At time 0, 1 mM isopropyl β-thiogalactoside or 50 ng/ml of anhydrotetracycline was added, and the uninduced control samples (solid lines) received an identical volume of growth medium. Viability was assayed by counting colonies grown overnight at 37 °C on M63 agar plates. For easier comparison all growth curves are normalized to the highest cell number in each panel.

**Fig. 3. Expression of C9 in dsb mutants and effect of N-acetylcysteine.** A, location of disulfide bonds in human C9 as published by Langewier et al. (27). E. coli dsbA mutant GJ73 transformed with pYW60 (●) or pYW51 (△) (B) and dsbB mutants NL100, transformed with pYW60 (●) (C), and YW10 transformed with pYW57 (●) or pYW58 (△) (D) were induced at −40 min with either anhydrotetracycline or isopropyl β-thiogalactoside to express periplasmic C9 (closed symbols) or cytoplasmic C9 (open symbols), and at 0 min NaC was added to promote disulfide bond formation. Normalized growth curves of uninduced cultures are shown in solid lines, and curves of induced cultures are in dashed lines. CFU values were determined as for Fig. 2.
Density of each fraction (recovered from the top) was determined (metrizamide solution of 1.29 g/ml density. After centrifugation the Crude lysates were equilibrated with metrizamide (final density, 1.27 g/ml) placed at the bottom of a centrifuge tube, and overlaid with a metrizamide solution of 1.29 g/ml density. After centrifugation the density of each fraction (recovered from the top) was determined (C) and analyzed by SDS-PAGE and Western blotting for the presence of C9 (D).

Periplasmic C9 is a soluble glycoprotein devoid of cellular toxicity; yet when it enters the periplasmic space of an E. coli cell it is converted from a protoxin to a toxin. This conversion is independent of the pathway by which C9

**DISCUSSION**

Native complement protein C9 is a soluble glycoprotein devoid of cellular toxicity; yet when it enters the periplasmic space of an E. coli cell it is converted from a protoxin to a toxin. This conversion is independent of the pathway by which C9
Complement-mediated Bacterial Killing

4691

enters the periplasm. Both, C9 shocked into the periplasmic space and C9 synthesized by expression from a plasmid and targeted to the periplasm via a signal sequence are toxic to the host cell. The toxic effects of plasmid-derived C9 were not caused simply by overexpressing a foreign gene because expression of C9 localized to the cytoplasm was not toxic. The lethal effects were also not due to jamming of the export pathway because of overexpression because the protein remained nontoxic when it was targeted to the periplasm of cells with impaired disulfide-bond catalysis (dsb mutants). In such cells the protein is translocated across the IM and becomes accessible in spheroplasts to externally added trypsin verifying its periplasmic location. The protein, however, is not present in a soluble form in the periplasm but is mostly aggregated, perhaps in so-called periplasmic inclusion bodies (30). A small percentage of translocated C9 is also still associated with the membrane. Wunderlich and Glockshuber (24) have shown that folding of a disulfide-bonded protein in the oxidative environment of the periplasm in well oxygenated cell cultures is influenced by addition of a thiol reagent such as NaC. When this catalyst was added to dsb mutants harboring C9 in their periplasm, it elicited an immediate loss of cell viability. We interpret this result to indicate that some cystine formation and therefore at least partial folding of the translocated C9 is necessary for toxicity. Because C9-mediated killing of bacteria is thought to occur by a single or dual hit process (31), the number of effective molecules is extremely low, and it is not surprising that NaC is useful in this process because very few C9 molecules need to form disulfide bonds and fold appropriately to evoke killing. The effectiveness of a few correctly folded C9 molecules is also demonstrated by the fact that transformation of wild type cells was not possible with plasmids in which expression is driven by promoters that are not tightly controlled.

Truncation of C9 indicated that the N-terminal 144 amino acids are not required for cytotoxicity and a fragment comprised of these residues accumulated in soluble form in the periplasm. The remainder, C9[145–538], however, is as toxic as the complete molecule when targeted to the periplasm. It comprised of these residues accumulated in soluble form in the periplasm and, as shown earlier (7), this fragment the complete molecule when targeted to the periplasm. The remainder, C9[145–538], however, is as toxic as the complete molecule when targeted to the periplasm. The remainder, C9[145–538], however, is as toxic as the complete molecule when targeted to the periplasm.

For C9-mediated killing of bacteria is thought to occur by a single or dual hit process (31), the number of effective molecules is extremely low, and it is not surprising that NaC is useful in this process because very few C9 molecules need to form disulfide bonds and fold appropriately to evoke killing. The effectiveness of a few correctly folded C9 molecules is also demonstrated by the fact that transformation of wild type cells was not possible with plasmids in which expression is driven by promoters that are not tightly controlled.

Truncation of C9 indicated that the N-terminal 144 amino acids are not required for cytotoxicity and a fragment comprised of these residues accumulated in soluble form in the periplasm. The remainder, C9[145–538], however, is as toxic as the complete molecule when targeted to the periplasm. It comprised of these residues accumulated in soluble form in the periplasm and, as shown earlier (7), this fragment the complete molecule when targeted to the periplasm.
13. Dankert, J. R., and Esser, A. F. (1987) *Biochem. J.* **244**, 393–399
14. Esser, A. F., and Sodetz, J. M. (1988) *Methods Enzymol.* **162**, 551–578
15. Dailey, F. E., and Berg, H. C. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 1043–1047
16. Bardwell, J. C., Lee, J. O., Jander, G., Martin, N., Belin, D., and Beckwith, J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 1038–1042
17. Jander, G., Martin, N. L., and Beckwith, J. (1994) *EMBO J.* **13**, 5121–5127
18. Derman, A. I., Prinz, W. A., Belin, D., and Beckwith, J. (1993) *Science* **262**, 1744–1747
19. Schneider, E. L., Thomas, J. G., Bassuk, J. A., Sage, E. H., and Baneyx, F. (1997) *Nat. Biotechnol.* **15**, 581–585
20. Levengood-Freyermuth, S. K., Click, E. M., and Webster, R. E. (1993) *J. Bacteriol.* **175**, 222–228
21. Jaskula, J. C., Letain, T. E., Roof, S. K., Skare, J. T., and Postle, K. (1994) *J. Bacteriol.* **176**, 2326–2338
22. Marvin, H. J., and Witholt, B. (1987) *Anal. Biochem.* **164**, 320–330
23. Tomlinson, S., Wang, Y., Ueda, E., and Esser, A. F. (1995) *J. Immunol.* **155**, 436–444
24. Wunderlich, M., and Glockshuber, R. (1993) *J. Biol. Chem.* **268**, 24547–24550
25. Thom, J. R., and Randall, L. R. (1988) *J. Bacteriol.* **170**, 5654–5661
26. Martin, J. L., Waksman, G., Bardwell, J. C., Beckwith, J., and Kuriyan, J. (1993) *J. Mol. Biol.* **230**, 1097–1100
27. Lengweiler, S., Schaller, J., and Rickli, E. E. (1996) *FEBS Lett.* **380**, 8–12
28. Rietsch, A., and Beckwith, J. (1998) *Annu. Rev. Genet.* **32**, 163–184
29. Stroud, R. M., Reiling, K., Wiener, M., and Freymann, D. (1998) *Curr. Opin. Struct. Biol.* **8**, 525–533
30. Betton, J. M., Boscus, D., Missiakas, D., Raina, S., and Hofnung, M. (1996) *J. Mol. Biol.* **262**, 140–150
31. Wright, S. D., and Levine, R. P. (1981) *J. Immunol.* **127**, 1146–1151
32. Gu, X., and Dankert, J. R. (1996) *J. Immunol. Methods* **189**, 37–45
33. Laine, R. O., and Esser, A. F. (1989) *Nature* **341**, 63–65
34. Tee, G. L., and Scott, G. K. (1980) *Infect. Immun.* **28**, 387–392
35. Kubens, B. S., and Opferkuch, W. (1988) *Zentralbl. Bakteriol. Mikrobiol. Hyg.* **A 276**, 52–65
Molecular Aspects of Complement-mediated Bacterial Killing: PERIPLASMIC
CONVERSION OF C9 FROM A PROTOXIN TO A TOXIN
Yunxia Wang, Edward S. Bjes and Alfred F. Esser

J. Biol. Chem. 2000, 275:4687-4692.
doi: 10.1074/jbc.275.7.4687

Access the most updated version of this article at http://www.jbc.org/content/275/7/4687

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 34 references, 15 of which can be accessed free at http://www.jbc.org/content/275/7/4687.full.html#ref-list-1