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Phagocytosis of *Vibrio cholerae* O139 Bengal by Human Polymorphonuclear Leukocytes

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Capsulated bacteria exhibit serum (complement) resistance and resistance to phagocytosis, which result in disseminated infections. *Vibrio cholerae* O139 strains possess a thin capsule and have been found to be partially serum resistant in a previous study. In the present study, compared to a standard capsulated *Klebsiella pneumoniae* strain, which showed total resistance to killing by phagocytosis, *V. cholerae* O139 strains were shown to be only partially resistant, with most strains showing <40% survival. These findings may explain the relative rarity of *V. cholerae* O139 bacteremia in cholera caused by this organism.

Vibrio cholerae O139 Bengal was identified as the second etiologic agent of cholera when it caused outbreaks of cholera in the Indian subcontinent in 1992 to 1993 and subsequently spread to several neighboring countries (1). *V. cholerae* O139 infection is now endemic in many countries of South and Southeast Asia. There is evidence to suggest that *V. cholerae* O139 derived from *V. cholerae* O1 El Tor through a process of genetic exchange whereby the genes responsible for O-antigen synthesis in *V. cholerae* O1 were replaced by novel genes which encode O139 antigen specificity (17). Even though there are striking similarities between *V. cholerae* O1 and O139, there are also differences, the most important of which is the possession of a polysaccharide capsule by *V. cholerae* O139 which is absent in *V. cholerae* O1 (10). In other bacteria that are capsulated, among other things, the capsule contributes to colony opacity, serum (complement) resistance, and resistance to phagocytosis (9, 13, 16). The twin properties of serum resistance and resistance to phagocytosis can give rise to disseminated infection (9, 13, 16). Similarly, *V. cholerae* O139 strains produce opaque colonies (10), are serum (complement) resistant (10, 12, 19), and cause septicemia at least in debilitated, immunocompromised patients (4, 8, 11). However, there are no data on phagocytosis of *V. cholerae* O139 by polymorphonuclear leukocytes (PMNL). This information is relevant to defining the potential of *V. cholerae* O139 to cause disseminated infection. We, therefore, studied the phagocytosis of the organism by human PMNL.

The bacterial strains used in this study are shown in Table 1. The *V. cholerae* O139 strains were clinical isolates cultured from the stools of cholera patients treated at the hospital of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), located in Dhaka, by standard methods (2). The strains were stored at −70°C in T1N1 broth (Trypticase, 1%; NaCl, 1% [pH 7.4]) with 20% glycerol. At the time of the study, the strains were grown on Luria agar (Difco, Detroit, Mich.), and their identities were reconfirmed. All of them produced opaque colonies (10) and possessed capsules, as evidenced by their lysis by vibriophage JA1, which has been shown to specifically lyse capsulated *V. cholerae* O139 (3). Strain 6A is a Tn*phoA* mutant of *V. cholerae* O139 AI-1837. The mutant has lost the abilities both to express a capsule and to produce lipopolysaccharide beyond the core oligosaccharide. It is also serum sensitive (killed by complement) (5). The mutant was donated by J. A. Johnson, University of Maryland School of Medicine, and Veterans Affairs Medical Center of Baltimore, Baltimore, Maryland. *Klebsiella pneumoniae* 11807/97 is a capsulated strain (serotype O1:K1) isolated from the blood culture of a child at the ICDDR,B hospital. *V. cholerae* O1 El Tor strains were from our culture collection.

Initially, three liquid media were evaluated for studies on resistance to phagocytosis: Casamino Acid yeast extract (CYE) broth (Casamino Acids, 3%; NaCl, 0.5%; yeast extract, 0.6% [pH 7.4]), AKI medium (Bacto Peptone, 1.5%; yeast extract, 0.4%; NaCl, 0.5%; $NaHCO_3$, 0.3% [pH 7.4]), and nutrient broth (NB) (Bacto Peptone, 0.8%; NaCl, 0.5%; beef extract, 0.5% [pH 7.4]). Single opaque colonies of *V. cholerae* O139 AI-1837 obtained after 20 h of growth at 37°C on Luria agar (Difco) were inoculated into 5 ml of each of the three media in glass test tubes and were incubated as still cultures at 37°C for 20 h. The bacteria were pelleted, washed once in 10 mM phosphate-buffered saline, pH 7.2, adjusted to approximately 10^8 CFU per ml by measuring the optical density at 600 nm in a spectrophotometer, and used for the phagocytosis assay. Phagocytosis was carried out as described previously (14). Briefly, blood was collected from nonimmune healthy adults, as judged by a lack of antibodies to the *K. pneumoniae* strain and *V. cholerae* O1 and O139 by the slide agglutination test, and PMNL were separated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient followed by dextran sedimentation. A mixture containing 120 μl of PMNL (10^7/ml), 5 μl of bacteria (10^8 CFU per ml), 5 μl of pooled human nonimmune sera (from five volunteers; this amount of serum was found optimal), and 70 μl of Hanks' balanced salt solution (Gibco, Grand Island, N.Y.) in a siliconized glass tube was incubated at 37°C for 1 h. A control tube containing 190 μl of Hanks' balanced salt solution, 5 μl of pooled sera, and 5 μl of bacteria was incubated in parallel. Bacteria surviving phagocytic killing were enumerated after lysis of the incubation mixture with deionized water and plating of various dilutions on gelatin agar and MacConkey agar (both from Difco). The percentage of inoculum surviving phagocytosis was calculated as (CFU in mixture

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with PMNL/CFU in mixture without PMNL) × 100. Each test was run in duplicate; each strain was tested three times, and the average count was taken.

In initial studies with three different media, the following survival rates were found for *V. cholerae* O139 AI-1837 upon phagocytosis: 78% in CYE broth, 57% in AKI broth, and 56% in NB. The noncapsulated 6A mutant was killed by opsonization with pooled sera; no killing of *K. pneumoniae* occurred upon phagocytosis after growth in any of the three media. Since the rates of killing were different after growth in different media, we investigated whether this was related to the amounts of capsular polysaccharide (CPS) produced in different media. CPS was purified by a method described previously (9) after strain AI-1837 was grown in the different media in 100-ml volumes. The concentration of CPS was determined by a phenol-sulfuric acid assay (6). The test was performed twice, and the average was taken. The CPS content was 45.5 ± 3.9 $\mu g/10^{10}$ CFU (mean ± standard error of the mean) when the strain was grown in CYE broth, whereas it was 8.5 ± 0.10 $\mu g/10^{10}$ CFU in AKI broth and 4.4 ± 2.15 $\mu g/10^{10}$ CFU in NB. This suggested that the degree of killing was related to the amount of CPS produced in different media during growth. Therefore, for subsequent studies, *V. cholerae* O139 strains were grown in CYE broth.

To determine whether opsonization was complement mediated, phagocytosis of strain AI-1837 was performed after heat inactivation of the serum at 56°C for 30 min. Heat inactivation resulted in 100% survival of the strain, which suggested that opsonization was complement mediated. To determine whether the classical or alternative pathway of complement was involved, serum was treated with 20 mM EGTA plus 5 mM $MgCl_2$ as described previously (7) to inactivate the classical-pathway activity. In both treated and untreated sera, the survival of strain AI-1837 was similar (70 and 75%, respectively). This suggested that the alternative pathway of complement was involved in opsonization.

The survival of *V. cholerae* O139 strains after phagocytosis is shown in Table 1. Compared to the control standard capsulated strain of *K. pneumoniae*, which showed 100% survival, most of the *V. cholerae* O139 strains showed <40% survival; however, two strains showed >60% survival. This variation may be due to a difference in the CPS content. The strains with higher survival may have a greater potential for causing septicemia. However, the overall data suggested that *V. cholerae* O139 strains are only partially resistant to phagocytosis. Both strains of *V. cholerae* O1 were extremely sensitive to phagocytic killing.

It has been demonstrated previously that the virulence of *K. pneumoniae* strains is due to the possession of a capsule. Typically, *K. pneumoniae* strains produce a thick capsule (18), and several serotypes, including the K1 serotype, are highly virulent and resistant to phagocytosis (15). Accordingly, the control strain of *K. pneumoniae* in our study was totally resistant to phagocytosis. Compared to this control strain, *V. cholerae* O139 strains were only partially resistant to phagocytosis. It has been demonstrated previously that *V. cholerae* O139 strains produce a relatively thin capsule (10), and the partial resistance to phagocytosis may be related to this finding. Similarly, compared to capsulated *K. pneumoniae* strains, *V. cholerae* O139 strains have been shown to be only partially serum resistant (12). In *V. cholerae* O139, the CPS and lipopolysaccharide are antigenically related, and the former is thought to be a polymerized form of the side chain of the latter (19).

It has been suggested that since *V. cholerae* O139 strains do not possess a thick capsule, the risk of disseminated disease in infected persons is relatively low. Nevertheless, the risk of disseminated infection may be greatest in persons with chronic underlying illness (10). In keeping with these predictions, only three cases of O139 septicemia have been recorded so far in the literature, and all of them occurred in persons with underlying problems (4, 8, 11). The relatively low incidence of septicemia due to *V. cholerae* O139 may be due to the low level of serum resistance of the strains, as described in a previous study (12), and to their low level of resistance to phagocytosis, as found in the present study.

TABLE 1. Phagocytosis of *V. cholerae* O139 and other bacteria

Bacterial strain	Mean (SD) % survival[a] after phagocytosis by PMNL
V. cholerae O139	
AI-1837	78 (1.2)
AI-7825	19.4 (1.2)
AI-11171	63 (1.4)
AJ-937	35 (1.7)
AJ-1619	35 (1.8)
AJ-35365	24 (2.7)
AK-5916	23.5 (2.7)
AK-40348	33.3 (2.1)
AK-17916	25 (1.6)
1005/96	25 (1.9)
11089/96	31 (1.1)
16960/96	33 (1.5)
10397/97	43 (1.6)
32422/97	27 (1.1)
33874/97	37 (1.3)
6A	0 (0)[b]
K. pneumoniae 11807/97	104 (1.1)
V. cholerae O1 El Tor	
19479	0.51 (0.2)
X-25049	0.15 (1.8)

[a] Values are geometric means from three experiments.
[b] Since all bacteria were killed during opsonization, phagocytosis could not be carried out under the conditions of the experiment.

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REFERENCES

1. **Albert, M. J.** 1996. Epidemiology and molecular biology of *Vibrio cholerae* O139 Bengal. Indian J. Med. Res. **104:**14–27.
2. **Albert, M. J., M. Ansaruzzaman, P. K. Bardhan, A. S. G. Faruque, S. M. Faruque, M. S. Islam, D. Mahalanabis, R. B. Sack, M. A. Salam, A. K. Siddique, M. Yunus, and K. Zaman.** 1993. Large epidemic of cholera-like disease in Bangladesh caused by *Vibrio cholerae* O139 synonym Bengal. Lancet **342:**387–390.
3. **Albert, M. J., N. A. Bhuiyan, A. Rahman, A. N. Ghosh, K. Hultenby, A. Weintraub, S. Nahar, A. K. M. G. Kibriya, M. Ansaruzzaman, and T. Shimada.** 1996. Phage specific for *Vibrio cholerae* O139 Bengal. J. Clin. Microbiol. **34:**1843–1845.
4. **Boyce, T. G., E. D. Mintz, K. D. Greene, J. G. Wells, J. C. Hockin, D. Morgan, and R. V. Tauxe.** 1995. *Vibrio cholerae* O139 Bengal infections among tourists to Southeast Asia: an intercontinental foodborne outbreak. J. Infect. Dis. **172:**1401–1404.
5. **Comstock, L. E., D. Maneval, Jr., P. Panigrahi, A. Joseph, M. M. Levine, J. B. Kaper, J. G. Morris, Jr., and J. A. Johnson.** 1995. The capsule and O-antigen in *Vibrio cholerae* O139 Bengal are associated with a genetic region not present in *Vibrio cholerae* O1. Infect. Immun. **63:**317–323.
6. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **3:**350–356.

7. **Fine, D. P.** 1977. Comparison of ethyleneglycoltetraacetic acid and its magnesium salt as reagents for studying alternative complement pathway function. Infect. Immun. **16:**124–128.
8. **Jesudason, M. V., A. M. Cherian, and T. J. John.** 1993. Blood stream invasion by *Vibrio cholerae* O139. Lancet **342:**431. (Letter.)
9. **Johnson, J. A., P. Panigrahi, and J. G. Morris, Jr.** 1992. Non-O1 *Vibrio cholerae* NRT36S produces a polysaccharide capsule that determines colony morphology, serum resistance, and virulence in mice. Infect. Immun. **60:**864–869.
10. **Johnson, J. A., C. A. Salles, P. Panigrahi, M. J. Albert, A. C. Wright, R. J. Johnson, and J. G. Morris, Jr.** 1994. *Vibrio cholerae* O139 synonym Bengal is closely related to *Vibrio cholerae* El Tor but has important differences. Infect. Immun. **62:**2108–2110.
11. **Khan, A. M., M. J. Albert, S. A. Sarker, M. K. Bhattacharya, and A. K. Azad.** 1995. Septicemia due to *Vibrio cholerae* O139 Bengal. Diagn. Microbiol. Infect. Dis. **22:**337–338.
12. **Meno, Y., and K. Amako.** 1994. The biological meaning of the capsule-like layer in *Vibrio cholerae* O139, p. 67–671. *In* Abstracts of the 30th Joint Conference: U.S.-Japan Cooperative Medical Science Program, Cholera and Related Diarrheal Disease Panel.
13. **Podschun, R., and U. Ullmann.** 1992. *Klebsiella* capsules type K7 in relation to toxicity, susceptibility to phagocytosis and resistance to serum. J. Med. Microbiol. **36:**250–254.
14. **Qadri, F., M. A. Haque, A. Hossain, T. Azim, K. Alam, and M. J. Albert.** 1993. Role of *Shigella dysenteriae* 1 slime polysaccharide in resistance to serum killing and phagocytosis. Microb. Pathog. **14:**441–449.
15. **Simoons-Smit, A. M., A. M. J. J. Verweij-van Vught, I. Y. R. Kanis, and D. M. MacLaren.** 1985. Chemiluminescence of human leukocytes stimulated by clinical isolates of *Klebsiella*. J. Med. Microbiol. **19:**333–338.
16. **Simoons-Smit, A. M., A. M. J. J. Verweij-van Vught, and D. M. MacLaren.** 1986. The role of K antigens as virulence factors in *Klebsiella*. J. Med. Microbiol. **21:**133–137.
17. **Stroeher, U. H., K. E. Jedani, B. K. Dredge, R. Morona, M. H. Brown, L. E. Karageorgos, M. J. Albert, and P. A. Manning.** 1995. Genetic rearrangement of the *rfb* regions of *Vibrio cholerae* O1 and O139. Proc. Natl. Acad. Sci. USA **92:**10374–10378.
18. **Sutherland, I. W.** 1978. Surface carbohydrate of the procaryotic cell. Academic Press, Inc., New York, N.Y.
19. **Waldor, M. K., R. Colwell, and J. J. Mekalanos.** 1995. The *Vibrio cholerae* O139 serogroup antigen includes an O-antigen capsule and lipopolysaccharide virulence determinants. Proc. Natl. Acad. Sci. USA **92:**11388–11392.