Emergence of Bactericidal and Opsonizing Antibody to *Vibrio vulnificus* following Bacterial Infection

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Virulent isolates of *Vibrio vulnificus* resist the bactericidal and opsonizing effects of normal human serum, in contrast to environmental isolates, which are highly serum susceptible. Immune responses to bacteremic *V. vulnificus* infections in human subjects have not been characterized. Serum from a patient who survived sepsis caused by *V. vulnificus* had substantial bactericidal and opsonizing immunoglobulin G (IgG) for his own bloodstream isolate. Killing was mediated by the classical complement pathway, whereas opsonization was effected by either the classical or the alternative pathway. IgG that reacted strongly with 55-, 58-, and 68-kilodalton outer membrane proteins was present in the patient's convalescent-phase serum but was absent from normal human serum. These findings suggest that humoral immunity to *V. vulnificus*, mediated by bactericidal and opsonizing antibody, emerges during infection and may be due, in part, to IgG directed against identifiable outer membrane proteins.

The development of immunity to *Vibrio vulnificus* has not been described. Earlier investigators observed that *V. vulnificus* resists the bactericidal effect of normal human serum (NHS) (5) and phagocytosis by polymorphonuclear leukocytes (PMN) (15); they also demonstrated an inverse relationship between the activation of complement pathways and the virulence of isolates (27). We (13) reported that blood culture isolates of *V. vulnificus* were completely resistant to the bactericidal effects of serum from normal adults and were poorly phagocytosed by normal human PMN after opsonization with this serum, in contrast to environmental isolates, which exhibited a 99 to 99.9% decline in viability and were readily phagocytosed by PMN under these same conditions. In the present paper, we describe in detail the presence of bactericidal and opsonizing antibody against *V. vulnificus* in the serum of a patient who was treated for and recovered from a serious infection and document the presence of immunoglobulin G (IgG) reactive with identifiable outer membrane (OM) protein fractions.

### MATERIALS AND METHODS

**Bacteria.** Five isolates of *V. vulnificus* were studied. V-1, V-2, and V-3 were cultured from the blood of patients who developed *V. vulnificus* sepsis after exposure to gulf waters in the Galveston, Tex., area; V-4 and V-5 are environmental isolates from Galveston Bay that were not known to have caused disease. Isolates V-2, V-3, V-4, and V-5 were previously characterized and identified as LC-1, LC-2, LE-8, and LE-10, respectively, in an earlier study (13).

**Serum.** On several occasions between 10 days and 24 weeks after the onset of illness, serum was obtained from a patient who survived overwhelming sepsis and cellulitis caused by isolate V-1. Pools of NHS were made, each by combining sera from 10 normal adults. Serum from a patient with severe combined immune deficiency syndrome (SCID), containing less than 15 mg of IgG per dl and no detectable IgM or IgA but possessing normal complement activity, was provided by William Shearer of Texas Children's Hospital. All serum samples were stored in 0.5-ml aliquots at −70°C. **Bactericidal assay** (13). About 5 × 10⁶ CFU of *V. vulnificus* in the logarithmic phase of growth were incubated per ml of phosphate-buffered saline at pH 7.4 (PBS) to which serum had been added; incubation was carried out in a shaking water bath at 37°C. At time zero and at 30- to 60-min intervals thereafter, aliquots were cultured quantitatively on blood agar plates. The classical complement pathway was selectively blocked with 0.01 M ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA)–10 mM MgCl₂ (3, 7), and the alternative pathway was blocked by heating sera to 50°C for 20 min (8); selective blocking of the alternative pathway was confirmed with appropriate controls (3, 7, 22). Consumption of complement by the alternative pathway was determined with 1 × 10⁵ to 5 × 10⁷ CFU of isolate V-1 with 10% serum in EGTA-dextrase-gelatin-Veronal buffer, with or without the addition of 100 mg of IgG per dl (21).

**Phagocytosis assay.** [³H]thymidine-labeled bacteria (13) were opsonized by incubating 5 × 10⁷ CFU/ml for 30 min in Hanks balanced salt solution containing 20% serum. Bacteria were then collected by centrifugation and suspended in Hanks balanced salt solution containing 0.1% gelatin. PMN were isolated from the peripheral venous blood of normal adults by dextran sedimentation and centrifugation over Ficoll-Hypaque (20). Phagocytosis was studied by adding 10⁶ PMN to 10⁵ CFU of *V. vulnificus* in a total volume of 0.4 ml and incubating the mixture with vigorous shaking at 37°C for 20 min. PMN and associated bacteria were separated by three centrifugations at 160 × g for 5 min. Vials with bacteria alone were included to determine a bacterium-alone background. The total number of bacteria present was determined by centrifuging some vials at 2,800 × g for 10 min. Pellets were suspended in a liquid scintillant (ACS; Amersham Corp., Arlington, Ill.). PMN-associated bacteria were reported as a percentage of the total counts per minute of [³H]thymidine-labeled organisms present during incubation after subtraction of the bacterium-alone back-
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Vibrio vulnificus (CFU/ml)

Time (Minutes)

0  60  120

FIG. 1. Bactericidal effect of various sera on isolate V-1. V-1 was incubated at 37°C in PBS with 20% convalescent-phase serum obtained 10 days after the onset of illness (A); 20% convalescent-phase serum that had been heated to 50°C for 20 min (B); 20% convalescent-phase serum plus MgCl₂-EGTA (C); MgCl₂-EGTA alone (D); or 20% NHS (E).

ground. An electron-microscopic examination verified that PMN-associated bacteria were contained within phagocytic vacuoles.

Isolation of IgG. IgG was isolated from serum by affinity chromatography with DEAE Affi-Gel Blue (Bio-Rad Laboratories, Richmond, Calif.). The eluate was lyophilized and suspended to a concentration of 1,000 mg of IgG per ml, as measured with radial immune diffusion plates (Meloy Laboratories, Springfield, Va.).

OM isolation. Bacterial cultures grown for 18 h in heart infusion broth were collected by centrifugation, washed once, and suspended in 10 mM N-2-hydroxyethylpiperazine-N' -2-ethanesulfonic acid (HEPES) buffer (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride and 100 Kallikrein inactivator units of aprotinin per ml. DNase and RNase (60 µg/ml each), lysozyme (100 µg/ml), and 0.5 M MgCl₂ (2 µl/ml) were added. OMs were obtained by French pressure cell disruption at 1,200 lb/in², followed by solubilization of the inner membrane with 1% sodium N-lauroyl sarcosinate (23).

SDS-PAGE. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), OM polypeptides were separated on linear 5 to 15% polyacrylamide gradient slabs gels or 10% polyacrylamide slab gels (1.5 mm) with 3% acrylamide stacking gels (17). OM-enriched samples were suspended in a sample buffer consisting of Tris base (63 mM), sodium dodecyl sulfate (2.5%), glycerol (12.5%), dithiothreitol (40 mM), and bromphenol blue (0.003%) and boiled for 2 min. Samples were loaded at 15 µg per well (gradient gels) or 150 µg per gel (10% gels). Electrophoresis was carried out at a constant current of 20 mA until the tracking dye reached the separating gel; the current was then increased to 35 mA for the remainder of the run. The apparent molecular weights of OM polypeptides were estimated from a calibration curve prepared with purified low-molecular-weight standards (Bio-Rad) and cytochrome c (Sigma Chemical Co., St. Louis, Mo.) included with each gel.

Immunoblotting (1, 4, 28). Proteins from SDS-PAGE were transferred to nitrocellulose membranes (0.45 µm, HAHY; Millipore Corp., Bedford, Mass.) with methanol-Tris-glycine buffer in a Bio-Rad electrophoretic transfer apparatus at 0.55 A for 90 min and at 0.85 A for the final 30 min. Nitrocellulose membrane blots were blocked for 1 h with 0.05% Tween 20 in PBS and incubated for 2 h with serum diluted 1:50 in PBST-0.03% Tween 20. After being washed, the nitrocellulose membrane sections were incubated for 1 h with the heavy- and light-chain-specific IgG fractions of goat anti-human IgG (Cooper Biomedical, Inc., West Chester, Pa.) diluted 1:2,000. The blots were washed and incubated with 125I-labeled protein A (specific activity, 37 mCi/mg; Amersham) for 1 h. In some experiments, alpha-chain-specific goat anti-human IgA or mu-chain-specific goat anti-human IgM (IgG fraction; Cooper) radiolabeled with 125I (18) was used to develop the blots. After incubation with radiolabel, the blots were washed, stained with amido black (26), dried, and exposed to Kodak X-Omat AR-5 film with a Lanex fine screen for 24 to 48 h.

RESULTS

Bactericidal effect. A 20% suspension of individual serum from any of 10 normal, healthy adults or either of two NHS pools had no bactericidal effect against human blood isolates V-1, V-2, or V-3 but killed >99% of the CFU of environmental isolates V-4 and V-5 during 60 min of incubation at 37°C. In contrast, a 20% suspension of convalescent-phase serum obtained 10 days, 5 weeks, or 24 weeks after the onset of illness killed 99 to 99.9% of V-1 in 60 min (Fig. 1). IgG extracted from convalescent-phase serum and added to NHS or SCID serum as a complement source had a similar bactericidal effect. Convalescent-phase serum was not bactericidal for V-2 or V-3, although it readily killed environmental isolates V-4 and V-5, as did NHS.

Selective blocking of the classical pathway eliminated the

| TABLE 1. Consumption of complement by V. vulnificus via the alternative pathway |
|---------------------------------|------------------|
| Serum                          | Complement consumption (°% decrease in rabbit 50% hemolytic complement) |
| SCID                           | 4.4              |
| NHS                            | 11.1             |
| CS                             | 63.7             |
| SCID + IgG from NHS            | 15.4             |
| SCID + IgG from CS             | 55.4             |
| NHS + IgG from CS              | 61.7             |

a CS, convalescent-phase serum taken 10 days after the onset of illness.
bactericidal effect of convalescent-phase serum; selective blocking of the alternative pathway had no effect (Fig. 1). Although the alternative complement pathway did not appear to contribute to bactericidal activity, two-thirds of the available complement was still consumed by this pathway when isolate V-1 was incubated with convalescent-phase serum or with NHS or SCID serum to which the patient’s IgG had been added (Table 1); only a modest degree of alternative pathway activation was seen with normal human IgG under the same circumstances.

Phagocytosis. The uptake of unopsonized isolate V-1, V-2, or V-3 by human PMN was minimal and increased only slightly after incubation with 20% NHS (Table 2). Phagocytosis of V-1 after opsonization with 20% convalescent-phase serum ranged from 87 to 93%, a result similar to that seen for phagocytosis of environmental isolates V-4 and V-5 after opsonization with 20% NHS. In contrast, opsonization of V-2 or V-3 with convalescent-phase serum did not stimulate greater uptake than that observed with NHS. Selective blocking of either the classical or the alternative complement pathway showed that phagocytosis of V-1 could be mediated by either pathway, although blocking of both pathways by EDTA abolished it (data not shown).

SDS-PAGE and immunoblotting. The five V. vulnificus isolates had generally similar OM protein patterns, although differences in the 22- to 28-kilodalton (kDa) range were noted (Fig. 2). Of interest was the absence of a 40-kDa protein in isolate V-1, even after additional passages in mice, and its presence in isolates V-2 and V-3; this protein has been said to be a marker for virulence in V. vulnificus (M. M. Carruthers, K. E. Jenkins, and W. J. Kabat, Clin. Res. 32:365A, 1984). The growth of V-2 and V-3 in the presence of 10 μg of clindamycin per ml led to a loss of this protein without altering the susceptibility to phagocytosis (data not shown).

TABLE 2. Opsonization of virulent and environmental isolates of V. vulnificus by convalescent-phase sera from an infected patient

<table>
<thead>
<tr>
<th>Isolate</th>
<th>% of available bacteria taken up by PMN opsonized with*</th>
<th>Nothing (unopsonized)</th>
<th>NHS</th>
<th>10-day CS</th>
<th>5-wk CS</th>
<th>24-wk CS</th>
</tr>
</thead>
<tbody>
<tr>
<td>V-1</td>
<td></td>
<td>23</td>
<td>31</td>
<td>87</td>
<td>93</td>
<td>93</td>
</tr>
<tr>
<td>V-2</td>
<td></td>
<td>15</td>
<td>32</td>
<td>25</td>
<td>28</td>
<td>20</td>
</tr>
<tr>
<td>V-3</td>
<td></td>
<td>19</td>
<td>37</td>
<td>31</td>
<td>37</td>
<td>43</td>
</tr>
<tr>
<td>V-4</td>
<td></td>
<td>45</td>
<td>84</td>
<td>86</td>
<td>ND*</td>
<td>ND</td>
</tr>
<tr>
<td>V-5</td>
<td></td>
<td>4</td>
<td>92</td>
<td>82</td>
<td>ND*</td>
<td>ND</td>
</tr>
</tbody>
</table>

* CS, Convalescent-phase serum.

Although the addition of immunoglobulin to NHS stimulated complement consumption by both pathways, consistent with recent observations on infrequently causes systemic infections and is often lethal when it does so. Pollak et al. (25) documented the presence of serum immunoglobulin that reacted with the homologous isolate after an orally acquired infection but did not examine functional (e.g., bactericidal or opsonizing) antibody. Isolate V-1 was isolated from a patient with typical V. vulnificus sepsis (2, 19). Although the patient’s preinfection serum was not available for study, the absence of bactericidal activity and the low degree of opsonizing activity against V-1 in the sera of 10 normal adults or two pools of NHS and the presence of these activities in the patient’s serum 10 days after the onset of infection, with persistence for at least 6 months, are consistent with the emergence of humoral immunity.

When convalescent-phase serum was incubated with V-1, complement was consumed and opsonization took place via either the classical or the alternative pathway. The addition of immune IgG to NHS stimulated complement consumption by both pathways, consistent with recent observations on
antigens and strain variations among V. vulnificus might be partly responsible.

Protective convalescent-phase antibodies directed against OM protein antigens have been found for Haemophilus influenzae type b (9), Campylobacter jejuni (21), and Neisseria gonorrhoeae (12) by use of electrophoresis and immunoblotting techniques similar to those used in the current study. Convalescent-phase serum obtained from our patient 17 days after infection contained IgG that reacted strongly with the 55-, 58-, and 68-kDa OM proteins of V-1, whereas NHS did not contain such IgG. It is possible that one of these proteins is the antigen to which bactericidal or opsonizing immunoglobulins or both are directed; IgG against the 55-kDa protein is most likely to be responsible, since antibacterial activity was already detected in the 10-day convalescent-phase serum, which contained this antibody, and neither antibacterial activity nor IgG to the 55-kDa protein was detected in V-2 or V-3. Verification of this hypothesis would require isolation of antibodies to the 55-kDa protein, perhaps with monoclonal techniques, as has been done for H. influenzae type b (10), and demonstration of the activity of those antibodies in functional assays. The relationship of any of these OM proteins to the common antigen of V. vulnificus, recently demonstrated by immunoelctrophoresis (24), remains to be determined.

A primary focus of this study was to examine the protein constituents of V. vulnificus to which the bactericidal and opsonizing activities might be directed. Recent research (16, 29) has suggested that a capsularlike material might be an important antigen of V. vulnificus and one against which protective antibodies might be directed. Since differences in the reactivity of our patient’s convalescent-phase serum may have been due to anticapsular antibodies, the presence of this material on our isolates was examined with ruthenium red staining (11). No capsule was conclusively demonstrated on any of the five isolates, which seemed to minimize a role for this material as an immunogen for our index patient or as a blocking factor in the case of serum reactivity with the virulent isolates V-2 and V-3. In addition, the serum from our patient, when tested for anti-surface antigen antibodies (16), did show an increase in titer over NHS. However, the assay was not specific for anticapsular antibody detection and may have reflected the binding of antibodies directed against other cell-surface-associated antigens, particularly in light of the inconclusive ruthenium red staining results.

In conclusion, our results reveal the presence of bactericidal and opsonizing antibody in the serum of a patient who was treated for and survived a serious V. vulnificus infection. IgG directed against an OM protein may have been responsible, exerting its effect by means of the classical complement pathway. Antibody to other cell components, e.g., lipopolysaccharide or capsule (16, 29), may also have been responsible. In any case, however, the failure of this convalescent-phase serum to kill other virulent isolates of V. vulnificus or to opsonize them for phagocytosis suggests that protection may reflect strain-related differences. Further study of the surface antigens of V. vulnificus may reveal the cellular basis for the virulence of some Vibrio species.

ACKNOWLEDGMENTS

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LITERATURE CITED


FIG. 3. Reactivity of human serum with electroblotted OM proteins from V-1. Transferred OM from a 10% slab gel were incubated with serum and then with goat anti-human IgG plus 125I-protein A. NS, Incubated with buffer instead of serum. Dates indicate the time that the convalescent-phase serum was obtained after the onset of illness. kD, Kilodaltons.
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