Preclinical immunoprophylactic and immunotherapeutic efficacy of antisera to capsular polysaccharide-tetanus toxoid conjugate vaccines of Vibrio vulnificus.

S J Devi, U Hayat, J L Powell and J G Morris Jr
Preclinical Immunoprophylactic and Immunotherapeutic Efficacy of Antisera to Capsular Polysaccharide-Tetanus Toxoid Conjugate Vaccines of *Vibrio vulnificus*

SARVAMANGALA J. N. DEVI,1 UNAIZA HAYAT,2 JAN L. POWELL,2 AND J. GLENN MORRIS, JR.2*

Division of Bacterial Products, Office of Vaccine Research and Review, Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, Bethesda, Maryland 20892,1 and Veterans Affairs Medical Center, and Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, Maryland 212012

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*Vibrio vulnificus* is an oyster-associated bacterial pathogen that causes life-threatening fulminating septicemia and necrotizing wound infections in humans. The capsular polysaccharide of *V. vulnificus* (VvPS) is critical for virulence. Previously we showed that active immunization of mice with a VvPS-tetanus toxoid (VvPS-TTa) conjugate vaccine conferred significantly higher protection against subsequent lethal challenge than immunization with VvPS alone. In the current study, we examined the utility of immunoprophylaxis or immunotherapy with hyperimmune antisera elicited by VvPS-TTa and VvPS-TTb conjugate vaccines prepared by different synthetic schemes. First we demonstrated that the Ribi adjuvant significantly enhanced the murine antibody response (P ≤ 0.02) to both conjugates. Subsequently, high-titered polyclonal antisera were raised to VvPS-TTa and VvPS-TTb conjugate vaccines by using Ribi adjuvant or Freund’s adjuvants. Antisera were observed to have protective effects when administered before and after acute lethal infection. All animals receiving prophylactic antisera intraperitoneally 24 h before lethal challenge with homologous carbotype 1 were protected, while 73 to 100% of control mice succumbed. Immunotherapy was also effective, with survival rates of 60 to 73% seen among mice when antisera were administered 2 h after bacterial challenge, at a time when symptoms of infection were already apparent. The protective effect of capsular antiserum appeared to be serotype specific. Antisera to the carbotype 1 VvPS-TTa vaccine did not confer cross-protection against lethal challenge with carbotype 2 *V. vulnificus* despite partial structural similarity and a weak serological cross-reaction between the two carbotypes. Immune globulins induced by a potential multivalent VvPS conjugate vaccine composed of clinically prevalent carbotypes may have utility in the management of *V. vulnificus* infections and deserve further evaluation.

*Vibrio vulnificus* is an estuarine, gram-negative bacterial pathogen belonging to the family *Vibrionaceae*. Unlike *V. cholerae* O1, *V. vulnificus* is encapsulated and invasive and causes fulminant primary septicemia and necrotizing wound infections in addition to gastroenteritis in humans (21). *V. vulnificus* specially affects subjects with liver disease, long-term alcohol abuse, diabetes mellitus, hemochromatosis, gastrointestinal disorders, and immunosuppression resulting from corticosteroid therapy, cancer, and AIDS (12, 14, 15, 32, 35). *V. vulnificus* infections have been reported in a subject with AIDS-related complex (3) and less frequently in apparently healthy subjects as well (14, 25). Infection is associated with consumption by susceptible hosts of raw shellfish containing the bacteria, especially oysters, or by contamination of preexisting wounds with seawater (21). The annual incidence of symptomatic *V. vulnificus* infection in U.S. coastal areas (Atlantic and Gulf coasts) approximates 0.6 per 100,000 (10, 12, 14). Cases have been reported not only from the United States but also from Korea, Japan, and Taiwan (4, 13, 23, 32).

Subjects with *V. vulnificus* septicemia often have a rapidly progressive, fulminant course (12, 14, 32), despite aggressive antimicrobial therapy and supportive care. Primary septicemia due to *V. vulnificus* is manifested by acute onset of fever and decreased blood pressure. Skin lesions, typically bullae, develop in more than half of patients with septicemia (12, 14, 23).

Fifty percent of patients with wound infections require surgical debridement or amputation (36). The mortality rate ranges from 55 to 79% and exceeds 90% for patients who become hypotensive within the first 24 h of hospitalization. In a summary of data from Florida cases (14), the mean time from hospital admission to death was 2 days. Subjects who survive frequently have long-term complications associated with multiple organ system failure and prolonged stays in intensive care units. Before culture results become available, it is often possible to make a presumptive diagnosis of *V. vulnificus* septicemia in a high-risk patient on the basis of the clinical presentation (hypotension and bullous skin lesions) and an appropriate epidemiologic history, i.e., consumption of raw oysters and exposure of a wound to seawater.

Several potential virulence determinants such as the *V. vulnificus* capsular polysaccharide (VvPS), a cytolytic hemolysin, an elastolytic protease, and the lipopolysaccharide are believed to play a role in pathogenesis (1, 8, 16, 31). Previous studies by us and other groups have established the critical role of VvPS in virulence and the less important role of cytolytin or elastase in pathogenesis (6, 18, 22, 37, 38). VvPS is known to confer resistance to phagocytosis (11, 17, 39) and to complement-mediated serum bactericidality (2, 11, 39). VvPS occurs in multiple carbotypes. Previously, we have identified 10 carbotypes in a limited number of clinical isolates obtained from different parts of the United States (9).

The high mortality, the severity of infections, and the rapidity with which septicemic patients die suggest the utility for vaccines or vaccine-derived prophylactic and therapeutic regi-
menses against V. vulnificus. We recently synthesized various prototype conjugate vaccines of V. vulnificus composed of carbotype 1 VPS covalently coupled to homologous cytolysin or elastase or to tetanus toxoid (TT), using two different synthetic schemes (6). The conjugate vaccines prepared following the carboxyl activation of the VvPS produced elevated capsular-specific antibodies and conferred 80% protection to actively immunized mice. In contrast, mice immunized with the unconjugated, T-cell-independent formulation of VvPS showed low levels of antibodies and 100% mortality (6), indicating its potential as a potential vaccine candidate. Cytolysin and elastase alone were not effective in protection.

The clinical management of V. vulnificus septicemia and wound infections involves appropriate antibiotic therapy and, in wound infections, removal of necrotic tissues. Passive immunization with hyperimmune globulins to capsular polysaccharides has been successfully used in the treatment of systemic infections caused by Haemophilus influenzae, Streptococcus pneumoniae, and Neisseria meningitidis (24, 30). Passive administration of antibodies is a potential adjunctive therapeutic option for the prophylaxis and treatment of V. vulnificus septicemia. In this report, we describe studies performed to evaluate the immunogenic potential of these conjugate vaccines in an adjuvant formulation admixed with the Ribi adjuvant.

V. vulnificus is a gram-negative rod-shaped organism, approximately 2 by 0.5 μm, which is usually isolated from the blood of patients with septicemia and was stored at −70°C in Luria (L) broth containing 50% glycerol.

Mice. Five- to six-week-old, female, outbred Swiss albino mice purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass., were used for all experiments. The animals were reared for about a week before the experiments were performed.

VvPS. VvPS of carbotype 1 and 2 were prepared and purified as described previously (9, 26, 27).

Conjugate vaccines. The synthesis, characterization, and composition of various VvPS conjugate vaccines prepared by using different synthetic schemes have been described previously (6). VvPS-TTa, VvPS-cytolysin, and VvPS-elastase were prepared by carboxyl activation of the VvPS, and VvPS-TTb was prepared by hydroxyl activation of the VvPS. All the conjugate vaccines were of carbotype 1 VPS.

Effects of the Ribi adjuvant on VvPS conjugate vaccines. Effects of the Ribi adjuvant on the immunogenicity of VvPS-protein conjugates were evaluated. The Ribi adjuvant consists of monophosphoryl lipid A (MLP) and trehalose dicorynomycolate (Ribi ImmunoChem Research, Inc., Hamilton, Mont.). Groups of mice were injected intraperitoneally (i.p.) with 5.0 μg of VvPS-TTa, VvPS-TTb, VvPS-cytolysin, and VvPS-elastase conjugate vaccines admixed in 50 μg of the Ribi adjuvant in saline. Mice were immunized on day 1 and boosted on day 21. Because of the availability of limited amounts of VvPS-cytolysin and VvPS-elastase conjugates, only one immunization was possible with these vaccines. Mice were bled on days 21 and/or 26, and the sera were analyzed for antibodies.

HYPERIMMUNE ANTISERA. Hyperimmune rabbit antisera to VvPS-TTa (enzyme-linked immunosorbent assay [ELISA] titer, 25,600) and hyperimmune murine antisera to TT were prepared by two subcutaneous or i.p. injections with Freund’s complete and incomplete adjuvants as described previously (9). Murine hyperimmune antisera to VvPS-TTa and VvPS-TTb were prepared by pooling serum sera from the experiment in which the Ribi adjuvant to an unrelated group B meningococcal polysaccharide (MenB PS) were similarly raised using a MenB PS-protein conjugate and the Ribi adjuvant (5).

Passive immunization. Two separate experimental designs were included, and all mice were immunized i.p. with VvPS-TTa or VvPS-TTb-induced antisera. Antisera to VvPS-cytolysin and to VvPS-elastase conjugate vaccines were not used in any protection experiments.

(i) Immunoprophylactic efficacy. Experiments 1 through 4 were performed to evaluate the immunoprophylactic efficacies of VvPS-TTa- and VvPS-TTb-induced antisera against carbotype 1 and 2 V. vulnificus septicemia by immunizing mice 24 h before challenge infection. Mice tested in experiments 1 and 3 were passively immunized i.p. with 150 μl of murine VvPS-TT conjugate-induced antisera. Each experiment included three control groups of mice, which received 150 μl of either saline or murine antibodies with specificity for TT or for an unrelated MenB PS. Mice tested in experiments 2 and 4 were passively immunized i.p. with murine antiserum to VvPS-TTa conjugate (200 μl), respectively. Control mice in these experiments received corresponding volumes of normal (nonimmune) rabbit or mouse serum. A group of eight mice was pretested for lack of serum sickness by injecting them i.p. with 200 μl of normal rabbit serum and observing them for 96 h.

(ii) Immunotherapeutic efficacy. Experiments 5 and 6 were performed to evaluate the immunotherapeutic efficacy of hyperimmune sera to both VvPS-TTa and VvPS-TT conjugate vaccines against carbotype 1 V. vulnificus by treating mice with the respective antiserum 2 h after lethal bacterial challenge. Mice tested in experiments 5 and 6 were treated with 150 μl of rabbit antiserum to VvPS-TTa conjugate vaccine and murine antisera to VvPS-TT conjugate vaccine, respectively. The control mice in both experiments received the same volume of normal, nonimmune rabbit or mouse serum.

Challenge infection of mice. Encapsulated and opaque variants of carbotype 1 or 2 V. vulnificus were harvested from a fresh L agar plate (6). The colonies were suspended in sterile phosphate-buffered saline (pH 7.4) and suspended at 107 CFU/ml volumes. Inoculum size was estimated by measuring the optical density at 600 nm of a log-phase culture and was confirmed by duplicate plating on L agar.

The challenge infection was performed I.p. as described previously (6). Mice in experiments 1 and 2 were challenged i.p. with ~15.50 lethal doses (LD50) (6 × 107 CFU) and ~5 LD50 (2.1 × 107 CFU) of carbotype 1 V. vulnificus, respectively, ~24 h after passive immunization. Mice in experiments 3 and 4 were challenged with ~1 LD50 (1.3 × 107 CFU) of carbotype 2 V. vulnificus ~24 h after passive immunization. Mice in experiments 5 and 6 and those mice that were injected with normal rabbit serum to observe for serum sickness were challenged with ~25 LD50 (9.9 × 107 CFU) of carbotype 1 V. vulnificus ~2 h before immunotherapeutic treatment with conjugate-induced antisera. Survival over challenge infection was chosen as the decisive criterion for protection, and mice were watched for death up to 76 h following the challenge.

Antibody analysis. A previously described ELISA was used to measure immunoglobulin G (IgG) and IgM capsular antibody levels in the individual and pooled sera. The linear dilution curves of individual murine sera were compared with the reference linear curve obtained with a pooled polyclonal murine antisera as described previously (6); the antibody levels were expressed as ELISA units per milliliter (mean ± standard error). The ability of VvPS-TTa-induced antisera to react with the carbotype 2 polysaccharide was also evaluated by ELISA (6, 9).

VvPS IgG and IgM levels (micrograms per milliliter) in the two murine hyperimmune sera used in protection experiments were quantitated by using VvPS-specific monoclonal IgG and IgM known concentrations of antibodies (24a) as reference standards in ELISA. The concentrations of specific antibodies in the monoclonal antibodies were quantitated with a commercial radiolabel immodification kit (Southern Biotechnology Associates, Birmingham, Ala.).

Statistical analysis. Differences in capsular antibody levels elicited by various VvPS-protein conjugate vaccines were determined by the two-tailed Student t test. The survival rates among mice in different study groups were compared by chi-square test 32 h following the challenge, after which no deaths occurred.

RESULTS AND DISCUSSION

In our previous study, we studied the immunogenicity of saline formulations of VvPS-TTa, VvPS-TTb, VvPS-cytolysin, and VvPS-elastase conjugate vaccines (6). In the current study, we evaluated the immunogenic potential of these conjugate vaccines in an adjuvant formulation admixed with the Ribi adjuvant. MPL is an attenuated lipopolysaccharide derivative with immunostimulating abilities (33). MPL has been administered to humans as a part of anticancer therapy (20) and also
against a 5-LD50 challenge with carbotype 1, the serum conferred 100% passive protection when administered, the serum conferred 100% passive protection when administered, the serum conferred 100% passive protection when administered.

The disease course of V. vulnificus septicemia in mice was noted. The i.p. administration of ~5 to 25 LD50 of carbotype 1 and ~1 LD50 of carbotype 2 V. vulnificus in mice resulted in a rapidly lethal septicemic infection. Within 2 h of infection, the mice manifested lethargy, ruffled fur, and ataxia. Almost all control mice died within 8 h following the infection irrespective of whether they were injected with saline or serum with specificity for an unrelated carbohydrate antigen (MenB PS) or for TT, before or after the infection. All eight mice that were observed for serum sickness after administration of nonimmune rabbit serum and then challenged with ~25 LD50 of carbotype 1 V. vulnificus also succumbed to septicemia (data not shown).

The concentrations of VvPS-specific IgG and IgM in murine hyperimmune antiserum to VvPS-TTa and VvPS-TTb conjugates were estimated to be 3,831 and 466 μg/ml and 1,500 and 274 μg/ml, respectively. Both VvPS-TTa- and VvPS-TTb-induced hyperimmune antiserum, at the doses used (41.0 to 93.0 μg of VvPS-specific IgM and 225 to 575 μg of VvPS-specific IgG), showed the potential to prevent mice from becoming infected with the homologous carbotype and also to prevent their death after a recent lethal bacterial challenge. A virtual 100% passive protection was conferred by VvPS-TTa- and VvPS-TTb-induced antiserum when given as immunoprophylactic agents 24 h before the lethal challenge (Table 3, experiments 1 and 2); 73 to 100% mortality was observed among control mice (P < 0.001 by chi-square test). Protection conferred did not require the simultaneous injection of antibodies and bacteria. Potentially, VvPS-binding antibodies act by potentiating phagocytosis and complement-mediated bacterial killing.

More striking was the survival of infected mice that were treated with the conjugate-induced antiserum after a lethal infection. When used as an immunotherapeutic regimen to treat mice with an established 2-h-old infection with ~25 LD50 of carbotype 1 V. vulnificus, VvPS-TTa conjugate-induced rabbit

### Table 1. VvPS antibody responses of mice immunized with various conjugate vaccines of V. vulnificus

<table>
<thead>
<tr>
<th>Vaccine (group)</th>
<th>IgG</th>
<th>IgM</th>
<th>IgG</th>
<th>IgM</th>
</tr>
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<tbody>
<tr>
<td>Expt A (saline)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VvPS-TTa</td>
<td>4.1</td>
<td>6.2</td>
<td>24.4</td>
<td>8.4</td>
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<tr>
<td>VvPS-T Tb</td>
<td>3.7</td>
<td>6.2</td>
<td>7.0</td>
<td>11.1</td>
</tr>
<tr>
<td>VvPS-cytolysin</td>
<td>6.7</td>
<td>7.8</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>VvPS-elastase</td>
<td>1.7</td>
<td>7.7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Expt B (Ribi adjuvant)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>VvPS-TTa</td>
<td>67.5</td>
<td>226.1</td>
<td>1,676.5</td>
<td>1,025.2</td>
</tr>
<tr>
<td>VvPS-T Tb</td>
<td>21.4</td>
<td>14.1</td>
<td>1,156.7</td>
<td>443.1</td>
</tr>
<tr>
<td>VvPS-cytolysin</td>
<td>28.2</td>
<td>148.6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>VvPS-elastase</td>
<td>26.8</td>
<td>729.6</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Swiss albino mice were immunized with various candidate vaccines of V. vulnificus as indicated, subcutaneously in saline (experiment A) or i.p. in the Ribi adjuvant (experiment B). Relevant results of experiment A are reproduced from a previous report (6) for comparison. VvPS-TTa and VvPS-TTb were synthesized by carboxyl and hydroxyl activations, respectively, of the VvPS. ND, VvPS-cytolysin and VvPS-elastase conjugates were not available in sufficient quantities for the second immunization. There was individual variation in antibody responses among mice of all groups (n = 8 to 10). Statistical analysis was by two-tailed Student t test. | t | P | t | P | t | P |
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<tbody>
<tr>
<td></td>
<td>a,b,c</td>
<td>0.0003; k,l versus e,f, and g,h;</td>
<td>0.002; w,x versus i,j and s,t, and 0.001; u,v versus p,c and y,z</td>
<td></td>
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<tr>
<td>Immunization 1</td>
<td></td>
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<td></td>
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<tr>
<td>Immunization 2</td>
<td></td>
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</table>

### Table 2. Immunotherapeutic efficacy of antiserum induced by VvPS-TT conjugate vaccines in mice

<table>
<thead>
<tr>
<th>Source of antiserum</th>
<th>Vaccine specificity</th>
<th>No. of mice</th>
<th>% Survival</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt 5 Rabbit</td>
<td>VvPS-TTa</td>
<td>11</td>
<td>72.7</td>
<td>0.01</td>
</tr>
<tr>
<td>Normal</td>
<td>10</td>
<td>20.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt 6 Mouse</td>
<td>VvPS-T Tb</td>
<td>10</td>
<td>60.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Normal</td>
<td>9</td>
<td>33.3</td>
<td></td>
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</table>

* The mice were protected i.p. with 150 μl of antiserum per mouse 2 h after i.p. challenge with 9.9 × 10⁷ CFU (~25 LD50) of carbotype 1 V. vulnificus. Mice were observed for death up to 76 h following the challenge.

| Statistical analysis (chi-square test) was for data obtained at 32 h following the challenge, after which no deaths occurred. |
The challenge, after which no deaths occurred, eversusf, g, h, 24 h before lethal challenge of mice with 1 LD₅₀ administration of VvPS-TTa-induced murine or rabbit antisera to V. vulnificus infection (27) between partial structural similarity and a weak serological cross-reactivity, and biologic activity is intriguingly complex. There reported signs of illness is very promising.

Micewereobserved for death up to 76 h following the challenge.

However, no statistical significance (P = 0.2) was achieved in the latter experiment compared with the survival rate among control mice (33%). It is most likely that an infection with a lower challenge dose would have produced a significant protective effect. Interestingly, the average survival in antibody-treated mice in experiments 5 and 6 was prolonged by 8 to 12 h (data not shown). The clinical relevance of reversal of progressive disease course and delay in death of mice following immunotherapy received after the establishment of a lethal infection (at a stage when animals were quite sick with visible signs of illness) is very promising.

The relationship between antigenic sharing, immunogenicity, and biologic activity is intriguingly complex. The reported partial structural similarity and a weak serological cross-reaction (27) between V. vulnificus carbotypes 1 and 2 prompted us to evaluate the ability of carbotype 1 conjugates to confer cross-protection against a lethal challenge with carbotype 2 V. vulnificus. At the amounts used in the current study, the i.p. administration of VvPS-TTa-induced murine or rabbit antisera 24 h before lethal challenge of mice with 1 LD₅₀ of carbotype 2 V. vulnificus had no protective effect on survival of mice, suggesting the lack of any such cross-protection (Table 3, experiments 3 and 4). There was 80 to 93% mortality among passively protected mice and 70 to 93% mortality among control mice that received saline or antisera to TT or to MenB PS. The lack of cross-protection may be due to the decreased density of protective determinants on carbotype 2 VvPS or masking of the protective epitope(s) by the extra sugars, α-2-acetamido-2,6-dideoxygalactopyranosyl and α-2-acetamido-2,6-dideoxymannopyranosyl, or may simply reflect the less than optimal quantity of antisera administered in the current study. The results suggest that experimentation using in vivo animal models is essential to correlate the biologic significance of serologic cross-reactivity between carbotypes and that serologic cross-reactivity is not a precise determinant of biological efficacy against cross-reacting carbotypes.

In conclusion, our results further support the importance of VvPS antibody-mediated protective immunity against V. vulnificus septicemia. The Ribi adjuvant significantly augmented the capsular antibody responses of four different VvPS-protein conjugate vaccines. A strong carbotype-specific passive protection was elicited in a murine model of V. vulnificus septicemia, using high-titered antisera raised in two different animal species to two VvPS-TT conjugate vaccines prepared by different synthetic schemes. The antisera conferred total protection to mice upon administration 1 day before lethal challenge infection with homologous carbotype 1 V. vulnificus. The finding that VvPS-TT-induced antisera administered after the onset of symptoms was able to reverse the disease course and extend the life of lethally infected mice suggests a potential therapeutic role for conjugate-derived immune globulins. The multiplicity of V. vulnificus carbotypes (7, 9) will necessitate the synthesis of multivalent conjugate formulations, and further studies will be required to estimate the minimum concentration of VvPS antibodies needed to confer protection in mice. Nonetheless, our results suggest that high-titered immune globulins (derived from plasma donors immunized with a multivalent VvPS conjugate) have possible utility both in passive immunophylaxis of targeted populations and as an adjuvant to therapy in persons with severe V. vulnificus infections.

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