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# Capsular Polysaccharide-Protein Conjugate Vaccines of Carbotype 1 *Vibrio vulnificus*: Construction, Immunogenicity, and Protective Efficacy in a Murine Model

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***Vibrio vulnificus* causes septicemia and wound infections in immunocompromised humans. The capsular polysaccharide of *Vibrio vulnificus* (VvPS) is critical for virulence. We synthesized conjugate vaccines of carbotype 1 VvPS under conditions and in formulations suitable for human use. Purified VvPS was conjugated to tetanus toxoid (TT) or to inactivated *V. vulnificus* cytolysin or elastase by two different schemes. All conjugates elicited elevated anticapsular immunoglobulin G (IgG) and IgM and antiprotein IgG responses in mice compared with saline placebo. The conjugates prepared through carboxyl activation of VvPS (VvPS-TTa, VvPS-cytolysin, and VvPS-elastase) were more immunogenic than the one prepared through hydroxyl activation (VvPS-TTb). The protective efficacy of conjugated and unconjugated formulations of VvPS and that of protein carriers were evaluated in a mouse septicemia model. Eighty percent of mice actively immunized with VvPS-TTa vaccine survived challenge with carbotype 1 *V. vulnificus*, while VvPS-cytolysin and VvPS-elastase conjugates conferred 44 and 40% protection, respectively. Control mice immunized with VvPS, cytolysin, or elastase alone, or saline only, showed 70 to 100% mortality. VvPS-TTa vaccine is nontoxic, immunogenic, and protective in mice.**

*Vibrio vulnificus*, first described in 1979 (7), is a gram-negative, halophilic, invasive, encapsulated bacterium which is being increasingly recognized as an etiologic agent of life-threatening primary septicemia and necrotizing wound infections in humans (1, 19, 30, 41). Infection occurs by contamination of wounds with seawater or by the ingestion of contaminated raw seafoods, particularly oysters (30).

*V. vulnificus* infections are often associated with underlying hepatic dysfunction, alcoholic liver disease, hemochromatosis (41), and long-term administration of corticosteroids (1, 16, 19, 20). *V. vulnificus* infections have also been reported in a subject with AIDS-related complex (3) and less frequently in apparently healthy individuals (19, 36). The clinical course of *V. vulnificus* septicemia is characterized by acute onset of fever, bullous and necrotizing skin lesions, hypotension, disseminated intravascular coagulation, and shock (29). Necrotizing soft tissue infections and cellulitis often require surgical debridement and may result in serious residual disabilities and amputation (46). Death due to primary septicemia usually occurs within 24 to 48 h despite appropriate antimicrobial therapy. The incidence of *V. vulnificus* infections has been estimated to be approximately 0.6 case per 100,000 population per year (14, 16, 19). Mortality rates exceed 50% in the United States, with 79% mortality reported in one large study in Korea (1, 16, 19, 34, 41); the mortality rate is 95% among patients who are hypotensive on hospital admission (19).

We and others (39, 50, 51) have demonstrated that *V. vulnificus* has a polysaccharide capsule (VvPS) which confers resistance to complement-mediated serum bactericidal activity (2, 15, 38) and resistance to phagocytosis (15, 23, 51). Encapsulation is critical for virulence. Strains with transposon mutations which result in loss of encapsulation have a 50% lethal dose ($LD_{50}$) that is more than 4 orders of magnitude higher than that seen with encapsulated, wild-type strains (50). The high mortality, severity of infection, and rapidity with which mortality occurs emphasize the need for effective approaches to prevention and management of *V. vulnificus* disease in high-risk populations (44). On the basis of the success of polysaccharide-protein conjugate vaccines in eliciting protection against other encapsulated pathogenic bacteria, we were interested in (i) determining if protection against *V. vulnificus* septicemia could be elicited in an animal model by VvPS-protein conjugate vaccines, (ii) assessing the relative immunogenicity of different protein conjugates synthesized by different conjugation methodologies, and (iii) evaluating the possible additive protective effects of conjugate vaccines composed of VvPS covalently coupled to *V. vulnificus* extracellular proteins.

## MATERIALS AND METHODS

**Chemicals and reagents.** Luria broth (L broth) was obtained from Difco Laboratories, Detroit, Mich. Cyanogen bromide, adipic acid dihydrazide (ADH), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC), thimerosal, bovine serum albumin, *p*-nitrophenyl phosphate, Brij 35, agarose, and Freund's adjuvant were from Sigma Chemical Co., St. Louis, Mo. Trinitrobenzene sulfonic acid was from Fluka Biochemika, Ronkonkoma, N.Y. Sterile water was from Biofluids, Inc., Rockville, Md. Sepharose 2B-CL was from Pharmacia Inc., Piscataway, N.J. Affinity-purified, alkaline phosphatase-labeled goat anti-mouse immunoglobulin G (IgG) and IgM antibodies were from Kirkegaard and Perry Inc., Gaithersburg, Md.

**Bacterial strain.** All studies were conducted with *V. vulnificus* MO6-24/O, isolated originally from a patient with septicemia (31). Bacteria were stored at −70°C in L broth containing 50% glycerol; working stocks were maintained by subculture on L agar at 30°C.

**VvPS and antisera.** Methods of extraction and purification of VvPS of strain MO6-24/O have been described previously (37). Rabbit and mouse hyperim-

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mune sera to VvPS and tetanus toxoid (TT), respectively, were produced as described before (5, 13).

**Protein carriers.** Purified TT, lot 39425, was purchased from Connaught Laboratories Inc., Swiftwater, Pa. Extracellular cytolysin and elastolytic protease of *V. vulnificus* were produced and purified in several steps as described previously (10, 21, 22). These protein preparations were homogeneous and contained only traces of endotoxin (10, 21, 22). The molecular weights of these proteins were determined by slab sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10, 22).

**Analytical methods.** Protein determination was done by Lowry's assay, using bovine serum albumin or TT as the standard; the residual lipopolysaccharide and nucleic acid were detected by the *Limulus* amoebocyte lysate assay (Sigma) and measurement of optical density at 260 nm ($OD_{260}$), respectively (5, 6). Hydrazide content of the derivatized VvPS was measured by the trinitrobenzene sulfonic acid assay, using ADH as the standard (5, 6). Since VvPS cannot be detected by the available colorimetric assays for sugars, it was monitored by gel diffusion and quantitated by rocket immunoelectrophoresis (45), using VvPS as the reference standard.

A nuclear magnetic resonance spectrum of the purified VvPS material was obtained to ascertain its structural features and purity (37). The partition coefficient and homogeneity of the VvPS and the conjugates were determined by gel filtration through a Sepharose 2B-CL column equilibrated with sterile water or 0.2 M sodium chloride (5, 6).

**Derivatization of VvPS.** Two schemes of derivatization were used. Scheme 1 was used to introduce ADH into VvPS following activation of its carboxyl groups with EDAC (5, 6). A 5.0-mg/ml solution of the VvPS in 0.2 M NaCl was reacted with 0.25 to 0.5 M ADH, using 0.1 M EDAC at pH 4.75 ± 0.25 for 3 h at room temperature. The reaction mixture was dialyzed thoroughly against sterile water and purified by passing through a Sepharose 2B-CL column equilibrated with sterile water. Fractions containing the derivatized VvPS as confirmed by gel diffusion with anticapsular hyperimmune serum and the trinitrobenzene sulfonic acid assay were pooled and freeze-dried.

Scheme 2 included introduction of ADH into VvPS following activation of VvPS with cyanogen bromide (5, 6). A 5.0-mg/ml solution of VvPS in 0.2 M NaCl was activated with a double amount (10.0 mg/ml) of cyanogen bromide at pH 10.5 for 8 min at 4°C. The reaction mixture was then combined with an equal volume of 0.5 M $NaHCO_3$, pH 8.5, containing 0.5 M ADH and tumbled at 3 to 8°C overnight. After extensive dialysis against sterile water, the solution was passed through a Sepharose 2B-CL column equilibrated with sterile water. The fractions containing the derivatized VvPS were collected, pooled, and freeze-dried.

**Conjugate synthesis.** A solution of the VvPS derivatized by either method, containing 1.6 to 10.0 mg/ml in 0.2 M NaCl, was mixed with an equal concentration of TT or cytolysin or elastase, and the pH was adjusted to 6.3. The reaction was initiated by the addition of 0.1 M EDAC and maintained at pH 6.3 ± 0.2 for 2.5 to 4.0 h at 4°C. After repeated dialysis against sterile 0.2 M NaCl at 3 to 8°C, the conjugate mixture was passed through a Sepharose 2B-CL column equilibrated with sterile 0.2 M NaCl. The fractions eluted in the void volume were confirmed to contain both VvPS and protein by chemical and immunological means and were pooled and preserved with 0.01% thimerosal at 3 to 8°C.

The VvPS-TTa and VvPS-TTb conjugates were synthesized by carboxyl and hydroxyl activation of VvPS, respectively.

**Residual toxicity.** A CHO cell assay (31, 48) was performed to evaluate the possible residual toxicity of VvPS-cytolysin and VvPS-elastase conjugates and of the inactivated *V. vulnificus* proteins prior to conjugation. VvPS-TTa conjugate and TT were included as controls.

**Mice.** Outbred, female Swiss albino mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.), 5 to 6 weeks old, were used for both immunogenicity and protection experiments.

**Immunization protocol.** Groups of 9 to 10 mice were injected subcutaneously two to three times, 15 days apart, with 5.0 μg of the VvPS in conjugated or nonconjugated form in saline. Mice were bled 7 days after each immunization (5, 6). Three groups of mice were injected with each of the three carrier proteins in concentrations equivalent to those in their corresponding conjugates. Uninoculated and saline-injected mice served as controls.

**Serological assays.** Conjugation of the derivatized VvPS with different carrier proteins was confirmed by performing gel diffusion with murine hyperimmune antisera to the purified VvPS and to the corresponding protein carrier (5, 6) (not shown).

Anticapsular IgG and IgM levels were measured in the individual serum samples from immunized and control mice by an enzyme-linked immunosorbent assay (ELISA) (5). Briefly, Immulon IV (Dynatech) plates were coated for 6 h with 10.0 μg of purified VvPS per ml in phosphate-buffered saline (PBS), pH 7.4. The plates were incubated at room temperature during the whole assay period. The plates were washed and blocked for 1 h with 1% bovine serum albumin in PBS containing 0.1% Brij 35. Eight serial serum dilutions in duplicate were made in PBS containing 1% newborn calf serum–0.1% Brij 35; the plates were incubated overnight. The washed plates were then incubated with alkaline phosphatase-labeled goat anti-mouse IgG or IgM reagent for 4 h. After thorough washing, the plates were incubated with *p*-nitrophenyl phosphate solution and were read at $OD_{405}$. VvPS antibody units were determined by using a reference curve of the standard serum obtained by linear regression of logit-log transformed data (4). The reference sera were assigned an arbitrary unitage of 1,000 ELISA units per ml.

A similar ELISA was done to analyze the sera for IgG antibodies to TT, cytolysin, and elastase (5). Because of the availability of limited amounts of cytolysin and elastase, parts of postimmunization serum samples from mice immunized with cytolysin and elastase in conjugated or nonconjugated forms were pooled and analyzed for IgG levels. Mice immunized with TT-containing vaccines were examined individually. Different murine hyperimmune polyclonal antisera to corresponding protein carriers were assigned an arbitrary unitage of 1,000 ELISA units per ml and used as reference standards.

**Active protection.** Mice were divided into 10 groups, A through J, each consisting of 9 to 10 mice except that group J contained 5 uninoculated control mice. Mice were immunized subcutaneously, 15 days apart, with different vaccines in saline. Groups A, B, C, and D were immunized three times with 5.0 μg of VvPS contained in VvPS-TTa, VvPS-TTb, VvPS-cytolysin, and VvPS-elastase conjugates, respectively. Groups E, F, and G received three injections of 3.7, 6.3, and 30.0 μg of elastase, cytolysin, and TT per mouse, respectively; these protein concentrations were chosen to match those received by the mice of groups D, C, and A as part of conjugate vaccines. Due to an inoculation error, mice of group I received only two injections of 5.0 μg of purified VvPS alone. Group H was injected with the diluent only. No adjuvants were used to immunize the mice included in the protection experiment.

A week after the last immunization, mice were challenged intraperitoneally with $1.8 \times 10^7$ CFU (~5 $LD_{50}$) of *V. vulnificus* MO6-24/O. Bacteria were suspended in sterile PBS, pH 7.4; the inoculum was given in a volume of 0.5 ml. Inoculum size was estimated by measuring the $OD_{650}$ of a log-phase culture, with counts confirmed by direct duplicate plate counts on L agar. Encapsulation of the inoculum was confirmed by the presence of the characteristic opaque colony morphology after incubation at 30°C on L agar.

*V. vulnificus* usually causes death in mice within 24 h of intraperitoneal challenge. In this study, mice were observed for 76 h, and mortality was recorded. Blood samples for antibody monitoring were collected by tail bleed prior to challenge and at the termination of the experiment 76 h after challenge. Sera were analyzed for VvPS antibody and also for corresponding anti-carrier protein antibody levels by ELISA.

**Statistical analysis.** Differences in antibody levels (mean ± standard error) between control and study groups were determined by two-tailed Student *t* tests. The chi-square test was used to analyze the survival data from the protection experiment.

## RESULTS

**VvPS, protein carriers, and conjugates.** The purified VvPS contained less than 1% protein and nucleic acid and <0.00003% of endotoxin by weight (37). The nuclear magnetic resonance spectrum of this material was similar to that previously reported by our group for the same strain (37); its repeating unit consists of three residues of 2-acetamido-2,6-dideoxyhexopyranose in the α-gluco configuration (QuiNAc) and one residue of 2-acetamido hexouronate in the α-galactopyranose configuration (GalNAcA) (37). The inactivated cytolysin and elastase showed molecular weights of 56,000 and 50,500, respectively, by SDS-PAGE (10, 21, 22). The characteristics of VvPS derivatized by both synthetic schemes, CHO cell assay titers obtained with the inactivated *V. vulnificus* proteins prior to and after conjugation, and the protein/polysaccharide ratio of various conjugates prepared by two different schemes are given in Table 1. All conjugates, irrespective of the scheme of conjugation followed, eluted in the void-volume fractions of the Sepharose 2B-CL column (Fig. 1).

**Antibodies.** There was considerable variation in individual antibody response among mice. Sera from mice immunized with saline contained 2.1 ± 0.2 and 9.5 ± 3.0 (mean ± standard error) U of IgG and IgM antibodies to VvPS per ml, respectively. The first immunization with VvPS alone did elicit low levels of IgG and IgM antibodies, which did not increase significantly after the second immunization (Table 2). The first immunization with the conjugates also induced no or low levels of IgG, which increased slightly with VvPS-TTb, VvPS-cytolysin, and VvPS-elastase following the second immunization and significantly with VvPS-TTa ($P = 0.009$ by the two-tailed Student *t* test). Compared with that of saline-injected mice, the best antibody response to all conjugates was observed follow-

TABLE 1. Composition and CHO cell assay titers of VvPS-protein conjugates of *V. vulnificus*

| Conjugate | % Derivatization of VvPS | Concentration (μg/ml) of: | | Protein/VvPS ratio[a] | CHO cell assay titer[b] |
|---|---|---|---|---|---|
| | | VvPS | Protein | | |
| VvPS-TTa | 3.0 | 24.0 | 144.2 | 6.0 | <1:2 |
| VvPS-TTb[c] | 1.1 | 44.0 | 419.7 | 9.5 | ND |
| VvPS-cytolysin | 3.0 | 64.1 | 81.0 | 1.3 | <1:2 |
| VvPS-elastase | 6.0 | 31.3 | 23.0 | 0.7 | <1:2 |

[a] The concentration of the reactants during the conjugation varied between 1.6 and 10 mg/ml.

[b] Highest twofold dilution which retained cytotoxicity. TT and inactivated cytolysin and elastase showed titers of <1:2, 1:16, and 1:32, respectively; the testing concentrations of the last two proteins were 5.7 and 1.6 mg/ml, respectively. ND, not determined.

[c] Activation of VvPS was performed through hydroxyl groups, using cyanogen bromide. The rest of the conjugates were composed of carboxyl-activated VvPS.

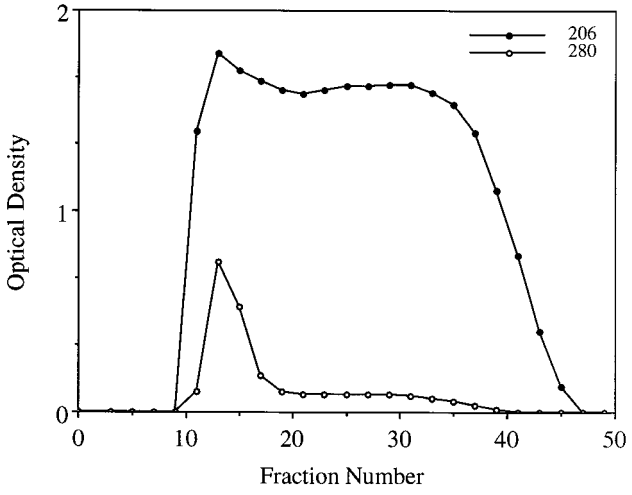


FIG. 1. Gel filtration profile of VvPS-TTa conjugate through a Sepharose 2B-CL column (1.5 by 30 cm) equilibrated in 0.2 M NaCl. The VvPS and TT in the fractions were monitored by measuring the $OD_{206}$ (●) and $OD_{280}$ (○), respectively. The fraction size was 1 ml. The conjugates eluting in fractions 11 through 19 were collected and pooled.

ing the third immunization ($P \leq 0.02$). The mean IgG level elicited by the VvPS-TTa conjugate after the third immunization was significantly higher than those elicited by the rest of the conjugates ($P \leq 0.02$). A rise in IgM level of threefold or more was elicited by all conjugates following the third immunization.

Elevated IgG antibodies to the respective carrier proteins were elicited by all conjugates (Fig. 2). Because of the difference in ratio of protein to VvPS in each of the conjugates, the amount of protein per dose varied, as did the antibody levels. The pooled postimmunization sera of mice administered VvPS-cytolysin and VvPS-elastase conjugates contained elevated IgG antibodies after the initial immunization, which increased fivefold or more after the third immunization. The third postimmunization rise in IgG response to cytolysin and elastase elicited by the corresponding conjugates was ~10-fold more compared with that elicited by the respective proteins alone. Mean anti-TT IgG levels of 11.2, 35.4, and 3.1 U/ml were measured in sera of mice immunized with VvPS-TTa, VvPS-TTb, and TT alone, respectively.

**Active protection.** A comparison of antibody levels and survival rate revealed that three immunizations of mice with 5.0 μg of VvPS-TTa per dose showed the highest capsular antibody levels and highest protection (80%) against an intraperitoneal challenge with ~5 $LD_{50}$ of *V. vulnificus* MO6-24/O (Fig. 3). This challenge dose killed 100% of mice that were uninoculated and those that were immunized with VvPS or cytolysin alone, 80% of mice injected with saline or TT, and 70% of mice immunized with elastase alone; these control mice showed mean serum capsular IgG and/or IgM levels of <5.2 and <15.2 U/ml, respectively (Table 2 and Fig. 3). VvPS-TTb was the least immunogenic conjugate and conferred protection to 22% of mice. VvPS-cytolysin and VvPS-elastase elicited mean serum VvPS IgG levels of 32 to 57 U/ml and protected 44 and 40% of mice, respectively. VvPS-TTa conferred significantly higher protection ($P \leq 0.02$ by the chi-square test) to mice than did VvPS-TTb, TT, VvPS, or saline alone. Most of the mice that succumbed to septicemia died between 8 and 16 h postchallenge; there was no death after 34 h following the challenge.

Prechallenge anticytolysin and antielastase IgG levels of 524 and 500 ELISA units per ml were attained in mice by three immunizations with VvPS-cytolysin and VvPS-elastase conjugate vaccines; the survival rates observed in these groups of mice were 44 and 40%, respectively. Group E, F, and G mice immunized with elastase, cytolysin, and TT alone elicited IgG

TABLE 2. VvPS antibody responses of mice[a] immunized with various vaccines of *V. vulnificus*

| Vaccine (group) | IgG/IgM level (ELISA units/ml, mean ± SE) | | | | | |
|---|---|---|---|---|---|---|
| | Immunization 1 | | Immunization 2 | | Immunization 3 | |
| | IgG | IgM | IgG | IgM | IgG | IgM |
| Saline (H) | ND | ND | ND | ND | 2.1 ± 0.2[b] | 9.5 ± 3.0[c] |
| VvPS (I) | 2.0 ± 0.4 | 6.7 ± 1.9 | 5.1 ± 0.9 | 15.1 ± 2.6 | ND | ND |
| VvPS-TTa (A) | 4.1 ± 1.3 | 8.2 ± 2.1 | 24.4 ± 6.1 | 8.4 ± 1.1 | 259.5 ± 84.8[d] | 79.0 ± 28.7[e] |
| VvPS-TTb (B) | 3.7 ± 0.9 | 6.2 ± 1.2 | 7.0 ± 2.5 | 11.1 ± 3.3 | 9.1 ± 3.1[f] | 22.6 ± 4.0[g] |
| VvPS-cytolysin (C) | 6.7 ± 4.8[h] | 7.8 ± 1.4 | 21.1 ± 17.7[h] | 15.4 ± 3.5 | 31.2 ± 9.2[i] | 41.4 ± 7.7[j] |
| VvPS-elastase (D) | 1.7 ± 0.3 | 7.7 ± 2.6 | 2.4 ± 0.4 | 11.4 ± 1.5 | 56.6 ± 20.8[k] | 24.0 ± 2.2[l] |
| TT (G) | 2.0 ± 0.4 | ND | ND | ND | 2.8 ± 0.3 | ND |
| Cytolysin (F) | 1.8 ± 0.3 | ND | 1.5 ± 0.3 | ND | 2.5 ± 0.4 | 9.8 ± 2.9 |
| Elastase (E) | 0.9 ± 0.3 | ND | 0.9 ± 0.2 | ND | 2.4 ± 0.6 | 10.6 ± 3.0 |

[a] Swiss albino mice were immunized with various candidate vaccines of *V. vulnificus*, as indicated, subcutaneously in saline. Group I mice received only two injections of VvPS because of a technical error. There was individual variation in antibody response among mice of all groups.

[b–g,i–l] Statistical analysis by the two-tailed Student *t* test: *e*, *g*, *j*, *l* versus *c*; and *d*, *k*, *i*, *f* versus *b*, $P \leq 0.02$.

[d,e,h,i] One or two mice in the group were high responders.

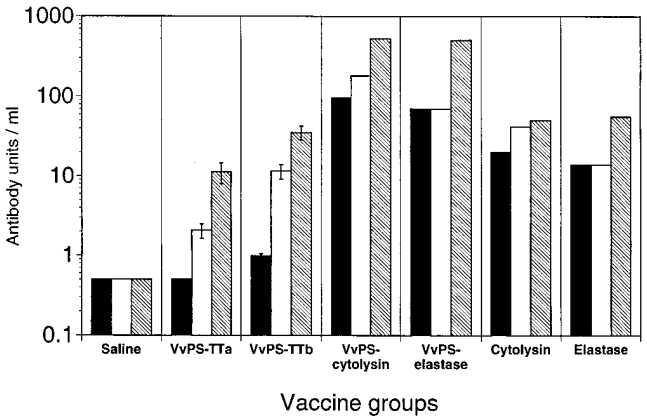


FIG. 2. Murine IgG antibody levels (units per milliliter, mean ± standard error) to corresponding carrier proteins following immunizations 1 (■), 2 (□), and 3 (▨) of various candidate vaccines of *V. vulnificus* administered in saline. The antibody levels (ELISA units per milliliter) depicted for cytolysin, elastase, VvPS-cytolysin, and VvPS-elastase conjugates were obtained with pooled sera of 9 to 10 immunized mice. Only post-immunization 3 sera of mice immunized with TT were analyzed for anti-TT IgG levels (data not shown).

levels of 50.0, 55.6, and 3.1 ELISA units per ml to the respective proteins and showed 70, 100, and 80% mortality, respectively.

Seventy-six hours following the intraperitoneal challenge, the VvPS IgG and IgM levels of VvPS-TTa-immunized mice decreased from 259.5 ± 84.7 to 165.3 ± 42.9 ($P = 0.37$ by the two-tailed Student $t$ test) and 79.0 ± 28.7 to 25.2 ± 4.8 ($P = 0.12$) U/ml, respectively.

## DISCUSSION

*V. vulnificus* is a highly virulent bacterial pathogen capable of causing fulminant septicemia and necrotizing wound infections in susceptible hosts. VvPS plays a critical role in the pathogenesis of *V. vulnificus* infections (39, 50, 51). We have found that some patients recovering from *V. vulnificus* septicemia have antibodies directed against the VvPS of the infecting strain (8), with the presence or absence of an antibody response dependent in part on the underlying degree of immunosuppression of the patient. There are insufficient data to comment on the protective efficacy of these anti-VvPS antibodies in humans. However, anticapsular antibodies are known to be protective against human systemic infections with other encapsulated bacterial pathogens such as group B *Streptococcus* types II and III (18), group A and C *Neisseria meningitidis* (9), and *Haemophilus influenzae* (32). There has been one study of active immunization of mice with formalin-killed *V. vulnificus* cells and cell extracts which implicated a "ruthenium red-staining surface carbohydrate" as the critical protective antigen (24); however, this study did not utilize purified capsular material, nor were efforts made to directly measure serum capsular antibody levels. In light of our recent work on purification and characterization of the structure of VvPS from strain MO6-24/O (37), we undertook a series of studies to evaluate if protective antibodies to *V. vulnificus* could be elicited by using VvPS-protein conjugate vaccines.

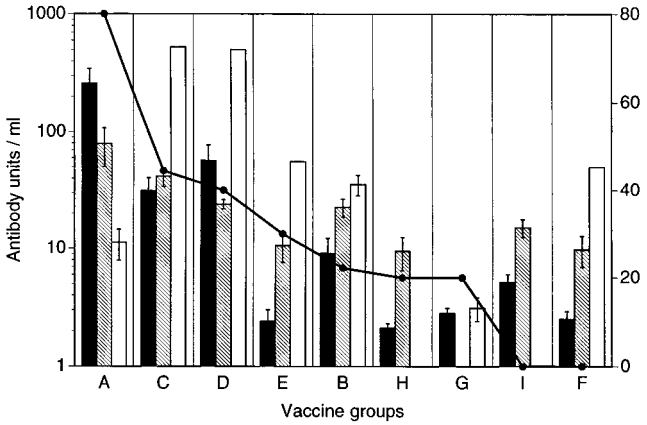


FIG. 3. Comparison of percent survival (●) of immunized and control mice (in decreasing order) with the postimmunization (prechallenge) anti-VvPS IgG (■) and IgM (▨) and antiprotein IgG (□) levels (units per milliliter, mean ± standard error, except for cytolysin and elastase and their conjugates). Vaccine groups: A, VvPS-TTa; B, VvPS-TTb; C, VvPS-cytolysin; D, VvPS-elastase; E, elastase; F, cytolysin; G, TT; H, saline; I, VvPS. VvPS IgM levels in the sera of group G mice and antiprotein IgG levels in the sera of mice from groups H and I were not measured. Percent survival (shown on the right vertical axis) was measured 36 h after challenge, after which no further deaths occurred.

*V. vulnificus* produces several proteins which are exported into the media during growth in vitro, including a distinctive cytolysin (10, 12, 25) and an elastolytic protease (21, 22). The purified cytolysin is lethal for mice in nanogram quantities (10) and has been shown to elicit skin lesions, similar to those found in patients with *V. vulnificus* septicemia (12). Antibodies to the cytolysin have been demonstrated in a patient recovering from *V. vulnificus* septicemia (11). However, strains of *V. vulnificus* in which the cytolysin gene has been mutated or deleted do not have decreased virulence in mice, suggesting that the cytolysin does not play a critical role in pathogenesis (47). Purified preparations of *V. vulnificus* elastase are also lethal for mice, although at microgram levels (21, 22). It has been suggested that the protease plays a role in utilization of heme by *V. vulnificus* (33); however, its importance in pathogenesis is not well defined, and again, there are reports suggesting that it is not essential for virulence (17, 31).

There has been considerable discussion about the use of homologous bacterial proteins as carriers for surface polysaccharides based on the hypothesis that an immune response to these proteins may increase the overall protective efficacy of the vaccine (4, 26, 28). We constructed prototype conjugate vaccines by covalent coupling of VvPS to the inactivated *V. vulnificus* cytolysin or elastase or to TT. Following three immunizations in saline formulation, the conjugate vaccines consisting of carboxyl-activated VvPS elicited elevated levels of capsular antibodies. Cytolysin and elastase elicited about 10-fold higher antiprotein IgG antibody levels in conjugate form than in the unconjugated form, similar to the observation made in rhesus monkeys with the outer membrane vesicle carrier in covalent conjugate formulation with group B *N. meningitidis* capsular polysaccharide (4).

The protective role in *V. vulnificus* septicemia of capsular antibodies elicited by T-independent (VvPS) and T-dependent (VvPS-TT conjugate) VvPS formulations was evaluated. Use of outbred mice in our studies may partly account for the variability in antibody response. The overall rate of survival was greater for all conjugate-immunized groups than for the VvPS- and protein-immunized or saline-injected mice (groups I, E, F, G, and H). The highest protection was conferred by VvPS-TTa. The least immunogenic VvPS-TTb conjugate was the least protective of all of the conjugates. Studies on the relative concentrations of VvPS antibodies in the sera of surviving mice showed that the VvPS antibody levels did not decrease significantly 76 h following the challenge infection. It

is most likely that the challenge infection resulted in the boosting of the capsular immune response in these mice.

The possible additive protective effect of VvPS-specific and cytolysin- or elastase-specific antibodies was evaluated by immunizing mice with VvPS conjugated to cytolysin or elastase. Cytolysin and elastase conjugated to VvPS were better vaccines in terms of both antibody production and ability to protect mice than were either cytolysin or elastase alone. These conjugates elicited elevated capsular and carrier-specific antibodies and protected 44 and 40% of mice, respectively. The difference in the degree of protection conferred by VvPS-TTa, VvPS-cytolysin, and VvPS-elastase could be due to one or two factors: (i) the latter two conjugates were synthesized at much lower concentrations than VvPS-TTa, and/or (ii) TT may be a better carrier protein than cytolysin and elastase. Although elastase alone conferred 30% protection, the difference was not statistically significant compared with the 20% protection observed in TT- or saline-injected mice. While the numbers involved are small, these data suggest that antibodies directed against either the cytolysin or the elastase provide no or minimal protection against subsequent challenge. As discussed above, this may reflect the relative lack of importance of these products in the pathogenesis of *V. vulnificus* septicemia in mice. Consistent with studies of immunity to *V. cholerae*, it also emphasizes the key role played by antibacterial as opposed to antitoxic immunity in development of protective antibodies in *Vibrio* species (27, 40).

Our studies were conducted with a single strain of *V. vulnificus*, MO6-24/O. We have subsequently found that *V. vulnificus* has multiple capsular types (13). The capsular type of strain MO6-24/O (designated carbotype 1) is significantly more common among clinical than environmental isolates (13); in studies involving over 150 environmental strains, we have yet to identify any carbotype 1 isolates. In much more limited studies, carbotype 1 together with carbotype 2 (which cross-reacts immunologically with carbotype 1) accounted for one-third of the patient isolates examined (13). The basis for the association between carbotype 1 and 2 strains and clinical illness is still not understood. Interestingly, however, carbotype 1 shares certain sugars with purified polysaccharide B of *Bacteroides fragilis* NCTC 9343, with which it cross-reacts immunologically (13). As recently reported by Tzianabos and colleagues (42, 43), the *B. fragilis* polysaccharide mediates formation of intra-abdominal abscesses on the basis of charge distribution; further studies on the possible role of the *V. vulnificus* polysaccharide as a biologic response modifier are in progress (35, 49).

From a practical standpoint, our data suggest that development of capsular polysaccharide-protein conjugate vaccines for *V. vulnificus* infections is possible. As there are multiple *V. vulnificus* carbotypes, such a vaccine regimen would need to be polyvalent, with further work required to define the most prevalent carbotypes for inclusion. With the identification by the U.S. Food and Drug Administration of patients with liver diseases, immune disorders including AIDS and cancer, gastrointestinal disorders, and diabetes mellitus as major risk populations (44), it seems that preinduction of capsular antibodies by active immunization of this target population with VvPS conjugate vaccines is feasible. Given the high mortality rates caused by *V. vulnificus* even with appropriate antimicrobial therapy and the rapidity with which septicemia culminates in fatality, it may also be useful to explore the efficacy of administering immune globulin containing high titers of VvPS antibodies in acute infections.

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