INFECTION AND IMMUNITY, Mar. 1986, p. 964–965
0019-9567/86/030964-02$02.00/0

Vol. 51, No. 3

Detection of Anti-*Vibrio vulnificus* Cytolysin Antibodies in Sera from Mice and a Human Surviving *V. vulnificus* Disease

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Received 9 September 1985/Accepted 27 November 1985

An enzyme-linked immunosorbent assay and a cytolysin neutralization assay were used to detect anti-*Vibrio vulnificus* cytolysin antibodies in sera from mice and a human that survived *V. vulnificus* disease. The detection of antibodies against the cytolysin indicated that the cytolysin is produced in vivo, and this observation is consistent with the hypothesis that the cytolysin is involved in the pathogenesis of *V. vulnificus* disease.

Vibrio vulnificus is a gram-negative, halophilic bacterium that is capable of causing rapidly progressing, life-threatening wound infections and septicemia in humans (1, 10, 14, 15). In addition, mice subcutaneously infected with *V. vulnificus* exhibit tissue damage and septicemia (2, 12) similar to that observed during human disease. We previously reported the purification and characterization of a *V. vulnificus* cytolytic toxin, and we are now studying the possible importance of the toxin in the pathogenesis of *V. vulnificus* disease. If the cytolysin is important in the pathogenesis of *V. vulnificus* disease, the cytolysin should be produced in vivo, and its production could be indirectly examined by determining whether anticytolysin antibody is produced during *V. vulnificus* disease. Other investigators have used enzyme-linked immunosorbent assays (ELISAs) (3, 6, 13) and toxin neutralization assays (4, 11) to detect antitoxin antibodies in sera from humans and experimental animals infected with *Pseudomonas aeruginosa*, *Campylobacter jejuni*, group A streptococci, and *Staphylococcus aureus*. The purpose of the present study was to use an ELISA and a cytolysin neutralization (CN) assay to determine whether anticytolysin antibodies could be detected in sera from mice and a human that survived *V. vulnificus* disease.

Experimental infections. Two groups of 6- to 8-week-old female CD-1 mice (50 mice per group) were injected subcutaneously with one 50% lethal dose (LD_{50}) of *V. vulnificus* E4125 (ca. 5×10^6 CFU) (7). Two weeks later, the survivors in one of the live-infected groups were injected subcutaneously with 2 LD_{50}s of the bacterium. We used the sequential challenge protocol to determine whether mice infected twice with the bacterium produce more anticytolysin antibody than mice infected once. Two groups of sham-infected control mice (25 mice per group) were injected subcutaneously with 1 LD_{50} equivalent of Formalin-killed *V. vulnificus* (9), and one of these groups was injected 2 weeks later with 2 LD_{50} equivalents of the killed bacterium. The sham-infected control mice were used to differentiate between anticytolysin antibody that might be produced in response to intracellular toxin present in the injected bacteria and anticytolysin antibody produced in response to toxin elaborated during in vivo replication of the bacteria. We observed previously that Formalin-inactivated cytolysin is antigenic (unpublished observation). A group of 25 normal mice was used as an untreated control group. The untreated mice and all surviving sham- and live-infected mice were exsanguinated 4 weeks after the first injection, and their sera were stored at −60°C until assayed by the ELISA and the CN assay.

ELISA. Wells of polystyrene 96-well microtest plates (Nunc; GIBCO Laboratories, Grand Island, N.Y.) were coated with purified *V. vulnificus* cytolysin (5) by incubation at 4°C for 18 h with 250 ng of cytolysin in Tris-buffered saline (TBS; 20 mM Tris, 500 mM NaCl [pH 7.5]). The wells were washed with TBS containing 0.05% Tween 20 (TBS-T), nonspecific binding sites were blocked by incubation with TBS-T containing 5% bovine serum albumin (ELISA grade; Sigma Chemical Co., St. Louis, Mo.), and the wells were washed with TBS-T. Serial twofold dilutions of the serum to be tested were prepared with TBS-T containing 1% bovine serum albumin, 50 μl of each dilution was added to wells treated as described above, the plates were incubated for 1 h, and the wells were washed with TBS-T. Portions (100 μl) of goat anti-mouse immunoglobulin G (IgG)-horseradish peroxidase (HRP) conjugate (diluted 1:3,000 with TBS-T–1% bovine serum albumin) or goat anti-human IgG + IgA + IgM-HRP conjugate (diluted 1:800) (both from Bio-Rad Laboratories, Richmond, Calif.) were added, the plates were incubated for 1 h, and the wells were washed twice with TBS-T and once with TBS. Substrate solution (0.5% Triton X-100, 0.02% H_2O_2, and 0.03 M *o*-phenylenediamine in 0.09 M Tris-citrate [pH 6]; 100 μl) was added, the plates were incubated for 5 min, the enzyme reaction was stopped by adding 100 μl of acid solution (0.1 M H_3PO_4, 0.02 M HCl), and the plates were read at 492 nm.

CN assay. Portions (1 μl) of the mouse sera were examined for CN activity by a previously published method (8), except that toxin-serum mixtures contained 3 hemolytic units and were incubated for 15 min at 25°C before erythrocytes were added.

Human serum. Serum samples from a human who had recovered from *V. vulnificus* disease were kindly supplied by Michael Hansen (Veterans Administration Medical Center, Houston, Tex.). The patient was a 56-year-old male alcoholic who had fished in Galveston Bay and eaten fresh crabs 2 days before presenting with sepsis and rapidly progressing cellulitis caused by *V. vulnificus*.

Detection of anticytolysin antibodies in mouse and human sera by the CN assay and the ELISA. Significantly more serum samples from mice that survived infection with 1 LD_{50} were positive for CN activity ($P = 0.002$; χ^2) than were samples from untreated and sham-infected mice (Table 1).

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TABLE 1. CN activity and anticytolysin ELISA titers of sera from mice surviving *V. vulnificus* disease

Treatment[a]	CN activity[b]	% Mice with positive ELISA titers[c]	Anticytolysin ELISA titers	
			Mean ± SD[d]	Range
Untreated	0 (0%)	0	<10	All <10
Sham-infected	4 (16%)	0	<10	<10–10
Live-infected	14 (58%)	67	733 ± 1,124	10–5,000

[a] Mice, except untreated controls, were injected subcutaneously with 1 LD_{50} of viable *V. vulnificus* E4125 (live-infected group) or 1 LD_{50} equivalent of Formalin-killed *V. vulnificus* (sham-infected group). All of the 25 untreated and the 25 sham-infected mice survived. Twenty-four of the 50 live-infected mice survived. Sera from surviving mice were assayed for CN activity and for anticytolysin ELISA titers.

[b] Number of sera positive for CN activity. Number in parentheses is the percent sera positive for toxin-neutralizing activity. Sera were considered positive if 1 μl of serum inhibited more than two-thirds of the hemolytic activity of 3 hemolytic units of cytolysin.

[c] Percentage of surviving mice whose sera had ELISA titers ≥40.

[d] The titer was the reciprocal of the highest serum dilution that resulted in an absorbance of 0.2.

Similar CN activity was observed in serum samples from mice that survived sequential challenge (data not shown).

Significantly more serum samples from mice that survived infection with 1 LD_{50} had positive ELISA titers ($P < 0.0001$; χ^2) than did samples from untreated and sham-infected mice (Table 1). Similar results were obtained with sera from mice that survived sequential challenge (data not shown). In addition, the mean ELISA titers of sera from live-infected mice were significantly higher ($P < 0.05$; analysis of variance and the method of Scheffe) than the mean ELISA titers of sera from untreated and sham-infected mice.

The ELISA also was used to test for anticytolysin antibody in sera from a human surviving severe *V. vulnificus* disease and in sera from six healthy human volunteers who, to the best of our knowledge, had never been infected with *V. vulnificus*. The ELISA titers of the patient sera were 320, 640, 640, and 1,280 at 10, 17, and 38 days and 6 months, respectively, after the onset of illness. The mean ELISA titer of the six normal human sera was 112 ± 39. Thus, the anticytolysin antibody titer of the 6-month serum sample was fourfold higher than the titer of the 10-day serum sample and was greater than eightfold higher than the mean titer of the normal sera. In addition, by day 17 after the onset of illness, the antibody titer of the patient serum was greater than fourfold higher than the mean titer of the normal sera.

One of several criteria that should be met to implicate an in vitro-produced bacterial toxin in the pathogenesis of disease by the bacterium is the demonstration that the toxin is produced in vivo during the disease process. Our detection of anti-*V. vulnificus* cytolysin antibodies in the sera of mice and a human surviving *V. vulnificus* disease indicates that the cytolysin, whose pharmacological activities are consistent with the grossly observable, pathological features of *V. vulnificus* disease (5), is produced during the course of the disease. This observation is consistent with the working hypothesis that the cytolysin is responsible, at least in part, for the severe tissue damage observed in *V. vulnificus* disease, and our observation has prompted additional studies to further evaluate the hypothesis. Experiments are in progress to determine (i) whether mice can be actively immunized against *V. vulnificus* disease by vaccination with the cytolysin and (ii) whether light and electron microscopic characterization of local tissue damage produced in mice injected with the cytolysin and in mice experimentally infected with the bacterium will reveal that cytolysin-induced damage mimics that produced by the bacterium.

This investigation was supported by Public Health Service grant AI-18184 from the National Institute of Allergy and Infectious Diseases.

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