Variability of Haemolysin(s) Produced by *Vibrio vulnificus*

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The peptide composition and antigenic cross-reactivity of partially purified and concentrated haemolysins of 16 strains of *Vibrio vulnificus* were examined by SDS-PAGE and immunoblotting analysis, using a monoclonal antibody (MAb), 6F8D, raised against the haemolysin. All strains produced a common peptide of 36 kDa and the MAb reacted with this peptide. In some strains, larger molecules, including a 56 kDa peptide, were produced, but the MAb did not react with this peptide. The haemolytic activity of the strains was effectively neutralized by the MAb, except in the case of strains producing the 56 kDa peptide. These findings indicate that the 36 kDa haemolysin is common to all 16 strains and that *V. vulnificus* can produce a second haemolysin which differs in molecular mass and antigenicity.

INTRODUCTION

Since *Vibrio vulnificus* (originally known as *Beneckeia vulnificus* and lactose-positive *Vibrio*) was reported by Reichelt *et al.* (1976) and Hollis *et al.* (1976) to cause severe wound infection and sepsis, virulence factors of this organism have been examined. It was reported that this bacterium could produce various kinds of extracellular products responsible for haemolysis, namely a phospholipase A2 and lysophospholipase (Testa *et al.*, 1984), and cytotoxin (Kreger & Lockwood, 1981; Gray & Kreger, 1985) or haemolysin (Tison & Kelly, 1984; Shinoda *et al.*, 1985). Gray & Kreger (1985) reported that purified cytotoxin of *V. vulnificus* had a molecular mass of about 56 kDa, as estimated by SDS-PAGE and by amino acid analysis.

In a previous study (Okada *et al.*, 1987), we described a monoclonal antibody (MAb) to the haemolysin produced from *V. vulnificus* strain FCC, which was different from the strain used by Gray & Kreger (1985) for the purification of the cytotoxin. This MAb recognized a 36 kDa peptide produced by strain FCC. These findings and those of Gray & Kreger (1985) suggested the existence of at least two distinct types of haemolysins in *V. vulnificus*. Here we report comparative studies by SDS-PAGE and immunoblotting to investigate possible heterogeneity in the molecular mass of the haemolysin.

METHODS

Bacteria. The strains of *Vibrio vulnificus* used are listed in Table 1. Strain FCC was used for the production of MAbs to the haemolysin. All 16 strains were maintained on slants of heart infusion agar (Eiken Kagaku Co.) supplemented with 3% (w/v) NaCl.

Preparation of haemolysin. Strains were cultured in heart infusion broth at 37 °C for 8 h, with shaking. After removing the organisms by centrifugation (16000 g, 30 min), ammonium sulphate was added to the pooled culture supernates (400 ml) to 40% saturation (282 g l⁻¹) and the preparation was then stirred overnight with a magnetic stirrer. The precipitate was recovered by centrifugation (16000 g, for 20 min) and dissolved in phosphate-buffered-saline (PBS: NaCl 8.0 g, KCl 0.2 g, Na₂HPO₄·12H₂O 2.9 g, KH₂PO₄ 0.2 g l⁻¹; pH 7.2).

Production of MAbs. The production and characterization of MAbs against the haemolysin produced by *V. vulnificus* strain FCC was as described by Okada *et al.* (1987). Briefly, BALB/c mice were injected subcutaneously

Abbreviations: MAb, monoclonal antibody; SRBC, sheep red blood cells.

Table 1. *Strains of V. vulnificus studied*

Strain	Source	Donor or reference
FCC	Blood and wound	Amako <i>et al.</i> (1984)
SAN	Blood	I. Azuma
MK	Knee joint fluid	T. Ishihara
FS	Blood	T. Ishihara
E-18	Sea water	I. Azuma
ATCC 27562	Type strain	Amako <i>et al.</i> (1984)
CDC B 3547	Leg wound	Shinoda <i>et al.</i> (1985)
B 1931	Blood	S. Shinoda
M 2799	Wound	S. Shinoda
371 Op	Sea food	Yoshida <i>et al.</i> (1985)
371 Tr	Sea food	Yoshida <i>et al.</i> (1985)
374 Op	Blood	Yoshida <i>et al.</i> (1985)
374 Tr	Blood	Yoshida <i>et al.</i> (1985)
L-1	Bone marrow	Yoshida <i>et al.</i> (1985)
L-180	Bone marrow	Yoshida <i>et al.</i> (1985)
C-4113	Unknown	S. Yoshida & Y. Mizuguchi

with 200 µg of the haemolysin on days 1, 7 and 14. Three days before killing the mice, a final booster injection of 100 µg haemolysin was given intravenously. Hybridomas were made by fusing the spleen cells with the P3U1 myeloma cell line, according to the procedure described by Harn *et al.* (1984). The antibody-producing cells were screened by the neutralization test for haemolysin. The hybridomas were injected into pristane-primed mice and the ascites was used as the source of MAb after precipitation with ammonium sulphate. The precipitate was dissolved in 10 ml PBS and dialysed. Clone 6F8D was used in the present study.

Haemolysin assay. The method was described in the previous paper (Okada *et al.*, 1987). Briefly, serially diluted haemolysin preparation (50 µl) was mixed in 96-well microtitre plates with 50 µl of a 1% (v/v) sheep red blood cell (SRBC) suspension in PBS. The plates were incubated at 37 °C for 1 h and the haemolysin titre was defined as the reciprocal of the highest dilution of haemolysin that showed complete haemolysis.

Neutralization of haemolysin by MAb. The method of the neutralization test was basically the same as that described by Okada *et al.* (1987). Briefly, twofold-diluted MAb (50 µl) was added to crude haemolysin preparation in a 96-well microtitre plate. The amount of the crude haemolysin was adjusted to the concentration that induced complete haemolysis. After incubation at 37 °C for 30 min, 50 µl of 1% (v/v) SRBC was added to the wells. The plates were incubated at 37 °C for 1 h and the inhibition titre was defined as the reciprocal of the highest dilution of MAb that inhibited the complete haemolysis.

SDS-PAGE. This was done by the method of Laemmli (1970), with the modifications described by Okada *et al.* (1987).

Immunoblotting procedures. These were as described by Okada *et al.* (1987).

RESULTS AND DISCUSSION

SDS-PAGE profiles of the haemolysins

Gray & Kreger (1985) reported that an extracellular cytotoxin of *V. vulnificus* had a molecular mass of about 56 kDa, which is larger than the haemolysin (36 kDa) produced by strain FCC used in this work. To clarify this discrepancy, the peptides of the haemolysin from various strains were analysed. Crude haemolysins were prepared from ammonium sulphate precipitates of culture supernates. The peptide profiles of the crude haemolysins produced by six strains of *V. vulnificus* from various sources are shown in Fig. 1. One peptide band at 36 kDa was common to all six strains (Fig. 1, arrowhead). In addition to this band, strains ATCC 27562 and SAN (which produced more proteins than the other strains) showed two more strongly stained bands at 56 and 60 kDa (Fig. 1, lanes 3 and 4). The 56 kDa peptide, which corresponded to the peptide reported by Gray & Kreger (1985), was undetectable or very faint in the other four strains. Strains FS and E-18 had one more band at 20 kDa (Fig. 1, lanes 6 and 7). These data suggest the possibility that two types of haemolysin are produced by *V. vulnificus*. Some strains produced both 56 and 36 kDa haemolysins and the others produced only the 36 kDa haemolysin. The significance of the low-molecular-mass peptide produced by strains FS and E-18 was not elucidated.

Fig. 1. SDS-PAGE of the extracellular haemolysin preparations from six representative strains of *V. vulnificus*. Samples were denatured under reducing conditions (2-mercaptoethanol in the solubilizing buffer). Lanes: 1, molecular mass markers (Pharmacia); 2, strain FCC; 3, strain ATCC 27562; 4, strain SAN; 5, strain MK; 6, strain FS; strain E-18. The amount of samples was 80 μ l per lane. The gel was stained with Coomassie brilliant blue R-250. The arrow indicates the 36 kDa band that was recognized by MAb 6F8D.

Fig. 2. Immunoblot analysis with MAb 6F8D. The haemolysin preparations were separated on SDS-PAGE and after electrophoretic transfer to nitrocellulose paper, the strip was incubated for 90 min with a 1 in 100 dilution of MAb 6F8D ascites. Lanes: 1, molecular mass markers that were transferred and stained with Amido black; 2-7, as in Fig. 1. The sheet was probed with horseradish peroxidase-conjugated goat anti-mouse immunoglobulins G.

Fig. 3. SDS-PAGE of the extracellular haemolysin preparations from 10 other strains of *V. vulnificus*. Lanes: 1, molecular mass markers (Pharmacia); 2, strain B 3547; 3, strain B 1931; 4, strain M 2799; 5, strain L-1; 6, strain L-180; 7, strain C-4123; 8, strain 371 Op; 9, strain 371 Tr; 10, strain 374 Op; 11, strain 374 Tr; the amount of samples was 100 μ l per lane. The gels were stained with Coomassie brilliant blue R-250. The upper arrowhead indicates the 56 kDa band reported by Gray & Kreger (1985), which is faint or undetectable in all lanes except lane 2. The lower arrowhead indicates the 36 kDa band, present in all lanes, that was recognized by MAb 6F8D.

Reactivity of anti-haemolysin MAb to the 56 and 36 kDa peptides

To investigate the antigenic cross-reactivity between the 56 and the 36 kDa peptides, their reaction with MAb 6F8D was examined by immunoblotting. Immunoperoxidase staining revealed that only the 36 kDa peptide reacted with the 6F8D antibody (Fig. 2). These data indicate that the 36 kDa peptide from the six strains shared a common antigenic epitope and that there was no cross-reactive epitope revealed by MAb 6F8D on the 56 kDa peptide.

Neutralization of haemolysin activity by MAb

To determine whether a peptide other than the 36 kDa species was responsible for haemolytic activity, neutralization tests of the haemolytic activity with MAb 6F8D were done. The haemolytic activity was effectively neutralized in four strains which did not produce the 56 kDa peptide, but was not neutralized in strains ATCC 27562 and SAN, which produced high-molecular-mass peptides (Table 2). This result indicates that strains ATCC 27562 and SAN produce another haemolysin which differs from that reactive to MAb 6F8D. This other haemolysin may be identical to the 56 kDa peptide reported by Gray & Kreger (1985).

To confirm whether the difference in the haemolysins was due to strain differences, a comparative study with 10 other strains was done. When crude haemolysin preparations obtained by ammonium sulphate precipitation were analysed by SDS-PAGE, all 10 strains were seen to produce the 36 kDa peptide, although the staining intensities differed among the strains (Fig. 3). Of these 10 strains, only strain B 3547 had a strongly stained band at 56 kDa (Fig. 3, lane 2). The 56 kDa peptide in the other strains was faint. These data indicate that *V. vulnificus* produces mainly 36 kDa haemolysin and some strains produce an additional haemolysin of 56 kDa.

Tison *et al.* (1982) classified *V. vulnificus* into two biogroups. They reported that *V. vulnificus* strains isolated from diseased eels differed from the type strain of the species (ATCC 27562), in respect of indole production. They proposed that strains phenotypically similar to the type strain be classified as biogroup 1 (indole production positive), and that strains phenotypically similar to those isolated from diseased eels (ATCC 33148) be classified as biogroup 2. All strains used in

Table 2. Neutralization of haemolysins from six representative strains of *V. vulnificus* by MAb 6F8D

Serially twofold-diluted monoclonal antibody (50 µl) was added to 50 µl haemolysin. The haemolysins were adjusted to the concentration that gave complete haemolysis. After incubation at 37 °C for 30 min, 50 µl 1% SRBC was added to the wells. The plates were incubated at 37 °C for 1 h. The inhibition titre was defined as the reciprocal of the highest dilution of antibody that inhibited haemolysis.

Source of haemolysin	Haemolytic-inhibition titre	
	Expt 1	Expt 2
FCC	2048	512
ATCC 27562	32	16
SAN	—	32
MK	4196	2048
FS	4196	2048
E-18	2048	1024

the present study produced indole (data not shown). These data suggest that there is little relationship between biogroups of the bacteria and types of haemolysins produced.

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