Monoclonal Antibodies against the Haemolysin of *Vibrio vulnificus*

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The extracellular haemolysin produced by *Vibrio vulnificus* strain FCC was partially purified from the culture supernate by sequential ammonium sulphate precipitation, gel filtration with Sepharose 4B, and DEAE-Sephacel ion-exchange column chromatography. Using this semi-purified haemolysin as the antigen, several monoclonal antibodies (MAbs) were established; they were all of the IgG2b class with lambda light chains. One representative MAb, 6F8D, completely neutralized the haemolytic activity and mouse lethal activity of extracellular toxin(s). In immunoblotting analysis of the peptides of the semi-purified haemolysin separated by SDS-PAGE, this MAb reacted, in particular, with a 36 kDa peptide. These findings suggest that the haemolysin is probably identical to the lethal toxin in the culture supernate of *V. vulnificus* strain FCC, which contained the 36 kDa peptide.

INTRODUCTION

Vibrio vulnificus is a halophilic bacterium causing gastroenteritis, wound infections, meningitis, myositis, pneumonia, or septicaemia in humans. Fulminating wound infections and septicaemia are often serious and the mortality rate is high in compromised patients especially in those with a liver cirrhosis or haemochromatosis (Blake *et al.*, 1980; Farmer, 1979; Hollis *et al.*, 1976; Tison & Kelly, 1984*b*). Putative virulence factors include extracellular cytotoxin (Gray & Kreger, 1985; Kreger & Lockwood, 1981), phospholipase A2 (Testa *et al.*, 1984), elastolytic protease (Kothary & Kreger, 1985), collagenase (Smith & Merkel, 1982), siderophores (Simpson & Oliver, 1983) and a surface antigen conferring antiphagocytic capacity (Amako *et al.*, 1984; Kreger *et al.*, 1984; Yoshida *et al.*, 1985). Pathological changes similar to those observed clinically, such as haemoconcentration, tissue necrosis or fatal septicaemia, also occur in experimental infections of animals (Bowdre *et al.*, 1981; Poole & Oliver, 1978).

Cytotoxin or haemolysin is apparently involved in the pathogenesis of *V. vulnificus* (Gray & Kreger, 1985, 1986). In cases of *Vibrio parahaemolyticus* infection, it was reported that thermostable direct haemolysin accounted for the mortality of the infected animal (Honda *et al.*, 1976). However, it is not known whether the haemolysin of *V. vulnificus* also has such lethal activity, or whether it is involved in the development of haemoconcentration.

Monoclonal antibodies (MAbs) are useful tools for analysing various bacterial toxins (Frank & Parker, 1984; Kamata *et al.*, 1985; Remmers *et al.*, 1982; Sheppard *et al.*, 1984). As MAbs bind to a single site on an antigen molecule, they are invaluable probes for studying structure–function relationships. In the present study, MAbs against *V. vulnificus* haemolysin were established and used to examine the relationship of the haemolysin to the lethal toxin produced by the bacterium.

Abbreviations: MAb, monoclonal antibody; MLD, minimal lethal dose; SRBC, sheep red blood cells.

METHODS

Bacterium and culture conditions. Strain FCC of *V. vulnificus* was used (Amako *et al.*, 1984). It was maintained on slants of nutrient agar supplemented with 3% (w/v) NaCl. For the production of haemolysin, heart infusion broth containing 0.5% NaCl was used. The bacterium was cultured aerobically at 37 °C for 8 h in a rotary shaker running at 200 r.p.m. (NBS, type G24, New Brunswick Scientific Co.).

Preparation of haemolysin. The culture supernate was obtained by centrifugation (16000 *g* for 30 min). Ammonium sulphate was added to the pooled culture supernate to 40% saturation (0.28 *g* ml⁻¹) and the preparation was then stirred overnight at 4 °C with a magnetic stirrer. The precipitate was recovered by centrifugation and dissolved in 10 mM-TE buffer (10 mM-Tris/HCl, 1 mM-EDTA, pH 7.0). The concentrated haemolysin (420 *mg* protein ml⁻¹) was applied to a column (2.6 × 90 cm) of Sepharose 4B gel (Pharmacia) and eluted with TE buffer at a flow rate of 15 ml h⁻¹. Pooled fractions from the gel filtration were applied to a DEAE-Sephacel column (1.3 × 45 cm, Pharmacia) equilibrated with 10 mM-Tris/HCl buffer, pH 7.0 (Tris buffer). After washing the column with the buffer, the adsorbed proteins were eluted with a linear gradient of 0 to 0.5 M-NaCl in Tris buffer followed by wash-out with 4 bed volumes (240 ml) of 1 M-NaCl in Tris buffer. Fractions were assayed for absorbance at 280 nm and for haemolytic activity. The fractions having haemolytic activity were used as the semi-purified haemolysin antigen for immunization.

Production of MAbs. Male 6-week-old BALB/c mice were injected subcutaneously with 200 µg of the semi-purified haemolysin suspended in Freund's complete adjuvant. On days 7 and 14 they were re-injected with 200 µg of the same antigen suspended in Freund's incomplete adjuvant. A final booster of 100 µg antigen in phosphate-buffered saline (8.0 *g* NaCl, 2.9 *g* Na₂HPO₄ · 12 H₂O, 0.2 *g* KCl, 0.2 *g* KH₂PO₄ l⁻¹, pH 7.2; PBS) was given intravenously 3 d before the cell fusion. Spleen cells were obtained on day 24 and were fused with mouse myeloma P3-X63-Ag-U1 (P3U1) cells at a ratio of 10:1, in a solution containing 35% (v/v) polyethylene glycol 1000, according to the procedure described by Harn *et al.* (1984). Fused cells were suspended in HT medium, comprising Dulbecco's modified Eagle medium (GIBCO) supplemented with 10% (v/v) NCTC109 (Whittaker, M. A. Bioproducts Inc.), 10% (v/v) NUSERUM (Collaborative Research Inc.), 2.5 mM-L-glutamine, 1 mM-cis-oxaloacetic acid (Sigma), 0.4 mM-sodium pyruvate (Sigma), 0.16 unit bovine insulin ml⁻¹ (Sigma), 50 µg gentamicin ml⁻¹ and 40.8 µg hypoxanthine/11.4 µg thymidine (HT) ml⁻¹. The cell suspension was then dispensed in 96-well tissue culture plates. HAT selection (Harn *et al.*, 1984) was done on days 1, 7 and 14 after cell fusion. Supernates of wells containing growing clones were tested on days 14 to 21 after fusion for MAbs, using enzyme-linked immunosorbent assay (ELISA) and neutralization of haemolysin, as described below. The antibody-producing cells were recloned by limiting dilution and hybridomas were injected into pristane-primed BALB/c mice. The ascites obtained from the mouse peritoneal cavity was precipitated with ammonium sulphate at 45% saturation. The precipitate was dialysed and each sample was adjusted to 10 ml of suspension. The class and subclass of MAbs were checked using isotype or subclass-specific antisera (Zymed Laboratories) by the Mono AB-ID EIA kit based on ELISA.

Haemolysin assay. This was based upon the assay described by Shinoda *et al.* (1985). Microtitre plates of 96 wells with conical bottoms containing 50 µl of a serial doubling dilution of the preparation to be assayed and 50 µl of a 1% (v/v) sheep red blood cell (SRBC) suspension in PBS were incubated at 37 °C for 1 h and kept at 4 °C for an additional 10 min to allow the unlysed SRBC to sediment. The haemolysin titre was defined as the reciprocal of the highest dilution of haemolysin giving complete haemolysis.

Neutralization of haemolysin by MAb. Serially twofold-diluted hybridoma culture fluids (50 µl) were added to 50 µl semi-purified haemolysin (5 µg protein ml⁻¹). After incubation at 37 °C for 30 min, 50 µl 1% (v/v) SRBC was added to the wells. The plates were incubated and stored as above. The haemolysis inhibition titre was defined as the reciprocal of the highest dilution of antibodies inhibiting haemolysis in the standard assay described above.

Neutralization of lethal activity by MAb. Neutralization assays were performed using eight to ten 5–7-week-old female ddy mice (Kyudo Experimental Animal Co.) per experimental group. Haemolysin at a dose of 1 MLD (minimal lethal dose, 100 µg per mouse) or 2 MLD was incubated with MAb (6F8D) diluted 1 in 10, 1 in 5 and 1 in 2 with PBS for 30 min at 37 °C; 0.4 ml of the mixture was then injected intravenously into mice and the mortality was observed for 3 d. In a passive transfer assay, mice was given intraperitoneally PBS-diluted MAb (6F8D), or P3U1-induced normal ascites, as the control. After 30 min, treated mice were challenged intravenously with semi-purified haemolysin at a dose of 1 or 3 MLD, and mortality was observed for 3 d.

SDS-PAGE. This was done by a modification of the method of Laemmli (1970). The samples to be analysed were boiled in solubilizing buffer [0.25 M-Tris buffer, 2% (w/v) SDS, 4% (v/v) 2-mercaptoethanol, 20% (w/v) glycerol and 0.1% bromophenol blue] for 3 min. Electrophoresis was performed at a constant current of 20 mA through a 4% (w/v) polyacrylamide stacking gel and a 12.5% slab separating gel. Protein bands were stained with 0.1% Coomassie blue/25% (v/v) 2-propanol/10% (v/v) acetic acid. In some experiments, 2-mercaptoethanol was omitted from the solubilizing buffer.

Immunoblotting procedures. After SDS-PAGE, proteins were transferred electrophoretically from the gel to nitrocellulose paper, using the method of Towbin *et al.* (1979). A constant potential of 30 V was applied to the gel-

nitrocellulose paper sandwich for 16 h in an electroblot buffer (25 mM-Tris/HCl, 192 mM-glycine, 20% (v/v) methanol; pH 8.3). The transferred proteins were stained with amido black. The paper was washed three times with PBS/Tween, incubated for 90 min at room temperature with MAb diluted in PBS/Tween, then washed three times with PBS/Tween and incubated with peroxidase-conjugated goat anti-mouse immunoglobulins (Cappel) diluted (1 in 800) with PBS/Tween. After 90 min incubation at room temperature and three washes, the blots were developed, using 4-chloro-1-naphthol as the peroxidase substrate.

RESULTS

The production of extracellular haemolysin was maximum when *V. vulnificus* FCC was cultured aerobically for 6 to 8 h at 37 °C in heart infusion broth containing 0.5% NaCl. Therefore, this condition was generally used for preparation of the haemolysin.

Partial purification of the extracellular haemolysin. The haemolysin was concentrated 46-fold from the culture supernate by ammonium sulphate precipitation. This concentrated haemolysin was eluted from the Sepharose 4B column forming two major protein peaks. The first peak, in the void volume fractions, contained slight haemolytic activity; most of the activity was recovered in the second major peak. For further purification, samples in the second major peak were applied to a DEAE-Sephacel column. Lethal activity and haemolytic activity were present in the fractions eluting at 0.25 to 0.4 M-NaCl. Samples of haemolysin at each purification step were analysed by SDS-PAGE (Fig. 1). Two major bands of 36 and 20 kDa, and many faint additional bands, were evident. During this step of purification, no significant change in the profile of the major peptide bands was observed, except that most of the minor peptides were eliminated in the final semi-purified haemolysin preparation. DEAE-Sephacel chromatography gave an increase in specific haemolytic activity of about 85-fold and a recovery of 13%. However, this haemolysin fraction contained the major 36 kDa peptide and there were still a few different peptide bands on the SDS-PAGE (Fig. 1, lane 4). The mobility in the gel of these peptides was not influenced by the presence of 2-mercaptoethanol in the solubilizing buffer. We used this semi-purified haemolysin fraction for further experiments.

Characterization of the semi-purified haemolysin. This haemolysin was active against erythrocytes from nine animal species: it was most active against sheep and mouse erythrocytes, and less active against chicken, rabbit and human erythrocytes. Heating at 56 °C for 30 min inactivated this haemolysin. The semi-purified haemolysin still possessed lethal activity in mice (MLD 100 µg protein per mouse). When mice were injected intravenously with 1 MLD of haemolysin, none survived for over 6 h. The haematocrit value at 5 h showed a prominent haemoconcentration.

Production of MAbs against haemolysin. Seven clones produced antibodies inhibitory to the haemolysin (Table 1). All were of the IgG2b subclass with lambda light chains. Clone 6F8D had the highest inhibitory titre (Table 1) and was used for further experiments.

Protective effect of MAb 6F8D against mouse lethal challenge with the haemolysin preparation. This was examined either by *in vitro* neutralization or by passive transfer of MAb to mice. When the haemolysin at a dose of 1 MLD was incubated with diluted (1 in 10, 1 in 5 or 1 in 2) MAb 6F8D, its lethal effect in mice was neutralized. MAb 6F8D diluted 1 in 5 or 1 in 2 also neutralized 2 MLD of haemolysin, but a 1 in 10 dilution only incompletely neutralized this amount of haemolysin (data not shown). In passive transfer experiments, the twofold-diluted MAb 6F8D sample conferred complete protection against challenge with 1 MLD of haemolysin and the protective effect of the MAb diminished with dilution. Similar patterns were observed even after challenge with 3 MLD of haemolysin (data not shown). Thus, we concluded that MAb 6F8D protected mice against the lethal activity exerted by the haemolysin.

Immunoblotting analysis. Reactivity of the MAbs to the peptides of the semi-purified haemolysin preparations was tested by immunoblotting analysis; a representative result with

Fig. 1

Fig. 2

Fig. 1. SDS-PAGE of the extracellular haemolysin preparations. Samples were denatured under reducing conditions (2-mercaptoethanol in the solubilizing buffer). Lanes: 1, molecular mass markers (Pharmacia); 2, ammonium sulphate precipitate (100 µg protein); 3, peak 2 of the Sepharose 4B gel filtration concentrate (100 µg protein); 4, DEAE-Sephacel eluate concentrate (80 µg protein). The gel was stained with Coomassie brilliant blue R-250.

Fig. 2. Immunoblot analysis: reactivity of MAb 6F8D was examined against haemolysin at different stages of purification. The haemolysin preparations were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose paper. The paper strip was then 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; 2-4, as in Fig. 1; blots were probed with horseradish peroxidase-conjugated goat anti-mouse immunoglobulins G.

Table 1. *Comparative ELISA reactivity and haemolytic inhibition titre of MAbs*

MAb*	ELISA reactivity (A_{490})†	Haemolytic inhibition titre‡
2F4H	0.398 ± 0.115	8
2F6A	0.332 ± 0.052	—
4G7G	0.498 ± 0.076	8
5D10A	0.352 ± 0.106	2
6F8D	0.405 ± 0.096	128
10D4C	0.300 ± 0.108	4
11F11D	0.310 ± 0.080	16

* All the MAbs were of the IgG2b-lambda subtype.

† The values represent means ± standard error of at least four experiments.

‡ Reciprocal of the highest dilution of MAb that blocked complete haemolysis. Control P3U1-induced normal ascites fluid had no inhibitory effect. —, Not determined.

the MAb 6F8D is shown in Fig. 2. Only one peptide on SDS-PAGE of the crude haemolysin reacted with 6F8D. MAbs other than 6F8D also reacted with the same band of protein (data not shown). These data suggest that the haemolysin is identical to the lethal product in the culture supernate of *V. vulnificus* and that the major antigenic epitope on the haemolysin was present in the 36 kDa peptide.

DISCUSSION

In the present study, several MABs were raised against semi-purified extracellular products of *V. vulnificus*. All the MABs studied clearly recognized a single peptide in the semi-purified extracellular products, as revealed by immunoblotting. One representative MAB, 6F8D, completely neutralized the haemolytic activity of the semi-purified toxin. MAB 6F8D also neutralized the lethal activity observed in mice. As an MAB is an antibody of defined specificity to a single epitope, these observations strongly suggest that the haemolysin is identical to the lethal toxin present in the culture supernate of *V. vulnificus*.

This observation supports evidence obtained by Gray & Kreger (1985) that the purified cytotoxin preparation has a haemolytic activity as well as lethal activity for mice, cytotoxicity for CHO cells, and vascular permeabilizing activity. Their cytotoxin had a molecular mass of about 56 kDa (estimated by SDS-PAGE and by amino acid analysis) and the peptide patterns of cytotoxin preparations in SDS-PAGE were influenced by the conditions used to denature the preparations. The haemolysin recognized by our MABs has a molecular mass of about 36 kDa (estimated by SDS-PAGE and by subsequent immunoblotting) and the peptide bands in SDS-PAGE were not influenced by denaturation, with or without 2-mercaptoethanol. However, similarities between their 56 kDa cytotoxin and our 36 kDa haemolysin include the following: (1) optimal conditions for haemolysin production were practically identical to those for cytotoxin production reported by Tison & Kelly (1984a); (2) the purified cytotoxin reported by Gray & Kreger (1985) was strongly hydrophobic and our haemolysin resembled their cytotoxin with regard to its behaviour during hydrophobic chromatography (i.e. eluting in the presence of 50% ethylene glycol: data not shown); (3) the pattern of sensitivity of erythrocytes from nine animal species to our haemolysin and the inactivation of the haemolysin by heating (56 °C, 30 min) were the same as observed by Gray & Kreger (1985).

These data suggest that these haemolytic materials produced by *V. vulnificus* are similar biologically active products but are heterogeneous, at least in molecular mass. There are at least three possible explanations for this hypothesis. One possibility is the production of two different haemolysins by a single strain, and a second is the production of different haemolysins from one strain to another. It might be possible for one strain of *V. vulnificus* to produce different kinds of haemolytic substances. *V. parahaemolyticus* produces various enzymes causing haemolysis, namely phospholipase A, lysophospholipase, glycerophosphorylcholine diesterase, and two kinds of direct haemolysins, one of which is heat-stable and the other heat-labile (Yanagase *et al.*, 1970). In the present experiments, we did not study the enzymic activity of the 36 kDa haemolysin. A third possibility is that the 56 kDa cytotoxin is cleaved by partial proteolysis to form the 36 kDa haemolysin. In this case, the epitope on the 56 kDa molecule might be inaccessible for our MAB because of the conformation of the molecule. Further study is required to prove that *V. vulnificus* has two different types of haemolysins. Immunoblotting analyses using various supernates produced by different *V. vulnificus* strains and our established MABs are now under way.

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