Production and characterisation of monoclonal antibodies to Verotoxins 1 and 2 from *Escherichia coli* of serotype O157:H7

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**Summary.** Fourteen hybridoma cell lines were isolated that produced monoclonal antibodies (MAbs) to purified Verotoxins 1 and 2 (VT1, VT2) of *Escherichia coli* of serotype O157:H7. Of these MAbs, eight were obtained by immunisation of BALB/c mice with purified VT1, and six were obtained from BALB/c mice immunised with purified VT2. With the exception of MAb 1C5, with a heavy chain of IgG\(_{2b}\) class, antibodies produced from mice immunised with heat-treated toxin were of IgM class. MAbs produced from mice immunised with heat-treated VT1 or VT2 reacted with both verotoxins in ELISA, and Western-blot analysis revealed that they reacted with subunit A and the A\(_1\) fragment of nicked subunit A of both toxins, but not with subunit B; furthermore, none of them neutralised Vero cytotoxicity or mouse lethality of either toxin. In contrast, MAbs produced from mice immunised with heat-treated and formalin-treated VT1 reacted in Western blots with subunits A and B of VT1 and subunit A, but not subunit B, of VT2, reacted in ELISA with VT1 only, and neutralised Vero cytotoxicity and mouse lethality of VT1 but not of VT2. Results indicate the existence of a common epitope on subunit A of VT1 and VT2 that is not responsible for the biological activity of these toxins, and that subunit B is essential for the biological activity of VT1. MAbs capable of reacting with both verotoxins from *E. coli* of serotype O157:H7 may be useful reagents for screening bacterial isolates capable of producing one or both of these toxins.

**Introduction**

Enterohaemorrhagic strains of *Escherichia coli* produce either or both of the verotoxins (VTs) VT1 or VT2 (Scotland et al., 1985), which are encoded by lysogenic phages 933J and 933W, respectively (Jackson et al., 1987). Both VT1 and VT2 consist of one subunit A and multiple copies of subunit B (Noda et al., 1987; Padhye et al., 1987, 1988 and 1989b; Yutsudo et al., 1987). Subunit B is presumed to bind specifically to the Vero cell-surface receptor and mediate uptake of the toxins, whereas subunit A causes inhibition of protein synthesis (Igarashi et al., 1987; Ogasawara et al., 1988) and subsequent cell death. Both toxins are cytotoxic to Vero and HeLa cells, produce pathological abnormalities in mice (Padhye et al., 1989a), rabbits (Keenan et al., 1986) or gnotobiotic piglets (Tzipori et al., 1986) and are lethal to mice (Strockbine et al., 1986; Padhye et al., 1989a).

Biochemical properties such as mol. wt, isoelec-

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either: (i) reacted only with subunit A of VT2 in immunoprecipitation assay, and did not neutralise the cytotoxicity of VT1 (Perera et al., 1988); or (ii) reacted in Western-blot analyses only with the A and A, subunits or with the B subunits of VT2 but not the A or B subunits of crude Shiga toxin (Downes et al., 1988).

We report here the production of MAbs to VT1 or VT2 that have substantially different properties from those of MAbs reported previously.

**Materials and methods**

**Purification of toxins**

VT1 was purified to homogeneity by a procedure reported before (Padhye et al., 1989b). Briefly, *E. coli* strain 932 of serotype O157:H7 was grown in mineral basal medium and a concentrated cell suspension was obtained by tangential filtration through a 0-5-μm Durapore (HVLP) cassette (Pellicon Cassette System, Millipore Corp., Bedford, MA). Cells were lysed by intermittent sonic disruption and, after removal of cell debris by low-speed (20 000g for 20 min) and high-speed (120 000g for 4 h) centrifugation, the VT1-containing fraction was precipitated between 30% and 60% (NH₄)₂SO₄. The toxin was purified by chromatographic separation on Sephacryl S-200 (Superfine) and Blue Sepharose Cl-6B, and chromatofocussing with Mono-P (FPLC) and Phenyl Superose (FPLC) (all products from Pharmacia).

For the purification of VT2, *E. coli* strain 932 was grown as described before (Padhye et al., 1986). Broth culture was filtered through a 0-5-μm Durapore (HVLP) cassette. Cell-free culture supernate was dialysed against 50 mM Tris buffer (pH 7-5) containing 0-3 M NaCl and concentrated with a polysulphone (PTC₃C) cassette followed by an Amicon concentrator with a PM-10 membrane. The toxin was purified by successive chromato-gramatic separation on Sephacryl S-200 (Superfine), QAE Sepharose (fast flow), Phenyl Superose (FPLC), Mono-P (FPLC), and Hydroxylapatite (Bio-Rad) as reported before (Padhye et al., 1987). Purity of the toxins was verified by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining.

**Immunisation**

Female BALB/c mice, 6–8 weeks old, in two groups were inoculated: either intraperitoneally with 2-5–4-5 μg of purified, heat-treated (90°C for 10 min) VT1 or VT2 mixed with an equal amount of Freund’s complete adjuvant; or subcutaneously with 2-0 μg of heat-treated (90°C for 10 min) and 1-0 μg of formalin-treated (formalin 2-7% v/v 37°C for 1 week) VT1 mixed with an equal amount of Freund’s complete adjuvant. After 4 weeks, mice were given, by subcutaneous injection, the same amount of heat-treated VT1 or VT2, or the mixture of heat-treated VT1 and formalin-treated VT1 mixed this time with an equal amount of Freund’s incomplete adjuvant. Thereafter, at 4 week intervals, the same amount of similarly treated toxin (VT1 or VT2) was administered intraperitoneally to mice until sera obtained by periodic bleeding from the tail vein gave titres of >200. Four days before cell-fusion, mice received a final intravenous “booster” injection with heat-treated toxin or with formalin-treated and heat-treated toxins. Thus, the same type of toxin was administered to mice throughout the immunisation series. Mice were killed for hybridoma preparation 5–6 months after the initial injection of heat-treated toxin, or 3 months after injection of heat-treated and formalin-treated VT1.

**Production of MAb**

MAbs to VT1 or VT2 were produced according to the procedure described by Campbell (1984) but with minor modifications. Splenocytes from hyperimmunised mice were fused with SP 2/0-Ag 14 myeloma cells by the use of 40% polyethylene glycol (mol. wt, 1300–1600) (J. T. Baker Chemical Co., Phillipsburg, NJ) and were grown in selective culture medium containing hypoxanthine, aminopterin and thymidine. Hybridomas were screened for production of antibodies reacting with VT1 or VT2 by an ELISA method described below. Positive hybridomas were subcloned twice by limiting dilution at 0.5 and 0.1 cell/well and tested for antibody production to VT1 or VT2. After the second cloning, isotypes of MAb were determined by ELISA.

**Solid-phase ELISA for anti-VT MAb**

Wells of enzyme immunoassay (EIA) plates (Gibco, Grand Island, NY) were coated with 0.5–0.7 μg of purified VT1 or VT2 in 50 mM carbonate buffer (pH 9-6). Plates were held at 4°C for 16 h and then washed five times with 0-1 M Tris (pH 9-0) containing 0.15 M NaCl. Hybridoma-culture supernate (0-1 ml) was added to each well, plates were incubated at 37°C for 1 h, washed five times with buffer A (0-05 M Tris, pH 7-4, containing 0-15 M NaCl, 20 mM ZnCl₂, 1-0 mM MgCl₂ and Na₂SO₄ 0-03% w/v) and nonspecific binding sites were blocked by treating with calf serum 50% v/v in buffer B (buffer A containing gelatin 0-25% w/v). After 10 min, 0.1 ml of goat anti-mouse immunoglobulin G (IgG) + IgM conjugated with alkaline phosphatase (Kirkegaard and Perry Labs Inc., Gaithersburg, MD), diluted 1 in 800 in buffer B containing calf serum 10% v/v, was added to each well and incubated at 37°C for 1 h. After five washes with buffer A, each well received 0-2 ml of phosphatase substrate (Kirkegaard and Perry Labs Inc.) in 1-0 M 2-amino-2-methyl-1-propanol, pH 9-9 (Sigma); absorbance was read after 1 h at 37°C and again after overnight incubation at room temperature.

**Determination of antibody class**

Isotypes of MAbs were determined by ELISA with class-specific antisera. Wells of EIA plates were coated
with purified VT1 or VT2 in 50 mM carbonate buffer (pH 9.6). After five washes with 50 mM Tris (pH 7.5) containing 0.15 M NaCl, wells received bovine serum albumin 5% w/v as a blocking reagent. After 1 h, 0.1 ml of supernate was added to each well. Incubation was continued for an additional 1 h at 37°C and rabbit antiserum, individually specific for mouse IgG1, IgG2a, IgG2b, IgG3, IgM, IgA, or 9 light chains (Mouse-types Isotyping Kit, Bio-Rad Labs, Richmond, CA), were added. After 1 h, 0.1 ml of alkaline phosphatase-labelled goat anti-rabbit IgG (diluted 1 in 800 in 50 mM Tris, pH 7.5, containing 0.15 M NaCl) was added to each well and incubated for 1 h at 37°C. Thereafter, phosphatase substrate (1.0 mg/ml) in 1.0 M 2-amino-2-methyl-1-propanol (pH 9.9) was added and absorbance (A405nm) was determined after 1 h at 37°C and after overnight incubation at room temperature.

**Western-blot analysis**

Purified VT1 or VT2 (3–5 µg) was electrophoresed on 5–30% gradient SDS-PAGE with reducing buffer (β-mercaptoethanol) to separate subunit A, fragment A1, of subunit A, and subunit B, or with non-reducing buffer (i.e., without β-mercaptoethanol) to separate subunits A and B. After electrophoresis, protein bands were transferred to nitrocellulose paper (pore size 0.2 µm, Schleicher and Schuell Inc., Keene, NH) by means of a Trans-blot apparatus, with minor modifications of the procedure described in the manufacturer’s instruction manual (Bio-Rad). After the nitrocellulose-gel sandwich had been electrophoresed for 15 h at a constant voltage of 60 V at 5°C, nitrocellulose paper was stained immunologically following incubation with 0.01 M Tris (pH 7.4) containing NaCl 0.9% w/v and Tween 20 (TST) 0.05% w/v for 2 h at room temperature (RT) in a sealed plastic bag kept on a rotating platform (Orbiton rotator, Boekel Industries Inc., Philadelphia, PA) to block nonspecific binding of antibodies. Antibody, diluted 1 in 10 in TST, was added to the plastic bag containing the nitrocellulose paper and incubated for 4 h at RT. After removal from the bag, the paper was thoroughly washed with TST and incubated with goat anti-mouse IgM (or IgG) radiolabelled with 125I (400 000 cpm/10 ml) (New England Nuclear) in a plastic bag rotated end-over-end for 4 h at RT. The paper was washed five times with TST and excess moisture was removed by blotting. After wrapping the plastic with the labeled paper, it was exposed to X-ray film (X-O mat XRP-1, Eastman Kodak Co., Rochester, NY) placed on an intensifying screen for 24 h at −70°C. The film was developed and protein bands on the nitrocellulose paper were detected after staining with Colloidal Gold Total Protein Stain (Bio-Rad).

**Results**

VT1 and VT2 of *E. coli* strain 932 from serotype O157:H7 were purified from cell lysate and culture supernate, respectively. SDS-PAGE analysis revealed that toxins were purified to homogeneity and that they resolved into subunits A and B after heat denaturation in a buffer containing SDS but without β-mercaptoethanol.

Both VT1 and VT2 were only partially inactivated before administration to mice; however, even after heating the toxins at 90°C for 10 min, an intraperitoneal dose of more than 5 µg (c. 10× LD50) was lethal to mice. Hence, less than 5 µg of
toxin was used for each injection. Fourteen hybridomas produced from three different fusions of mouse splenocytes with myeloma cells were identified and cloned: (i) from a mouse immunised with heat-treated VT1 only, five hybridomas, designated 4C9, 5E1, 6F1, 10D12, and 10F4 and producing antibodies reactive with VT1 and VT2, were obtained; (ii) from a mouse immunised with heat-treated VT2 only, six hybridomas, designated 1C5, 2D5, 1E1, 3E4, 4F10 and 8H10 and producing antibodies reactive with VT1 and VT2, were obtained; (iii) from a mouse immunised with heat-treated VT1 and formalin-treated VT1, three hybridomas, designated 3C10, 1OD11, and 9C9, were obtained (table). All MAbs from hybridomas of the first fusion were composed of IgM heavy chain and κ light chain, whereas those from the third fusion were of IgG heavy chain and κ light chain. Of six MAbs generated against VT2, only those designated 1C5 were of IgG heavy chain; all other MAbs to VT2 were of the IgM class (table). Light chains of all MAbs to VT2 were of the κ class.

MAbs were also assayed for their ability to react in ELISA with VT1 and VT2, to neutralise Vero-cell cytotoxicity and mouse lethality, and to bind in Western blots with subunits A or B of the toxins. Partial purification of antibodies from ascitic fluid used for these studies was essential because crude ascitic fluid was toxic to Vero cells and produced high levels of non-specific reactions in Western blots. All of the IgM-class antibodies were partially purified to the point at which Vero-cytotoxicity factor was removed from ascitic fluids. IgG antibodies from ascitic fluid were purified to homogeneity.

None of the MAbs produced from mice immunised with heat-treated VT1 or VT2 only, neutralised, either individually or in various combinations, Vero cytotoxicity or mouse lethality of purified VT1 or VT2 (table). Each of three MAbs, produced from mice immunised with heat-treated and formalin-treated VT1, neutralised completely Vero cytotoxicity and mouse lethality of purified VT1 but not VT2 (table). These results indicate that antibodies from mice immunised with only heat-treated toxin were reacting with an epitope of VT1 or VT2 that is composed of a protein domain not responsible for Vero cytotoxicity.

All MAbs to VT1 and VT2, produced from mice immunised with only heat-treated toxin, reacted with both VT1 and VT2 in a non-competitive ELISA. However, antibodies produced from mice immunised with heat-treated and formalin-treated VT1 reacted with VT1 only.

Western-blot analyses were done to define subunit specificities of MAbs. VT1 and VT2 are composed of subunit A and multiple copies of subunit B, the former often nicked into fragments A1 and A2 which are linked by a disulphide bridge. In the presence of β-mercaptoethanol, SDS-PAGE (5–30% gradient gel) of VT1 produced three bands

<table>
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<tr>
<th>Clone</th>
<th>Immunising antigen*</th>
<th>Isotype†</th>
<th>Verotoxin subunit specificity‡</th>
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<th>Neutralisation of toxicity to Mice</th>
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* The immunising antigens were VT1 or VT2 treated only by heating except that, where indicated*, both heat-treated and formalin-treated preparations of VT1 were used.
† For all MAbs, the light chains were of the κ class.
‡ Determined by Western-blot analysis of SDS-denatured toxins.
corresponding to subunit A (c. 31 Kda), fragment A$_1$ of subunit A (c. 27 Kda), and a mixture of subunit B and fragment A$_2$ of subunit A (c. 4 Kda) (fig. 1); tested similarly, VT2 resolved into subunit A (c. 32 Kda), fragment A$_1$ of subunit A (c. 27 Kda), and a mixture of subunit B and fragment A$_2$ of subunit A (c. 6 Kda). When β-mercaptoethanol was omitted from the disruption buffer, both toxins appeared after SDS-PAGE as two bands corresponding to subunits A and B (data not shown). Subunits separated on polyacrylamide gel were transferred to nitrocellulose paper and stained with Colloidal Gold All Protein Stain to confirm the successful protein transfer (fig. 1). The proper combination of voltage, buffer concentration, and duration of protein transfer was necessary for transfer of the toxin subunits and their fragments to nitrocellulose paper. Variations in these experimental conditions resulted in either unsuccessful transfer of subunit A on to the nitrocellulose paper or migration of subunit B beyond the nitrocellulose paper.

Reaction of the toxin subunits with rabbit polyclonal antibody raised against purified VT2 is shown as a positive control in fig. 2. Anti-VT2 reacted with subunit A, with A$_1$ fragment and with the combination of subunit B and the A$_2$ fragment of both VT1 and VT2 (lanes 1 and 2, respectively, fig. 2). However, binding of anti-VT2 with subunit A and the A$_1$ fragment of VT1 was substantially less than that observed with VT2 (fig. 2).

Western-blot analysis of MAb 2D5 with the toxins is shown (fig. 3); this MAb, generated against VT2, reacted with subunit A and the A$_1$ fragment of both VT1 and VT2 (lanes 1 and 2, respectively, fig. 3). It did not, however, react with either subunit B or with the A$_2$ fragment of either toxin. The other MAbs produced from mice immunised with heat-treated VT1 or VT2 only, gave identical results in the assays performed (table).

In contrast, MAbs 3C10 and 9C9, produced from mice immunised with both heat-treated and formalin-treated VT1, reacted with subunit A and the A$_1$ fragment of VT1 and with subunit A of VT2; it also reacted strongly with the subunit B of VT1 but not with that of VT2 (fig. 4; the Western blot of VT1 and VT2, separated in the presence of nonreducing buffer, i.e., without β-mercaptoetha-

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**Fig. 1.** Resolution of VT1 (5 µg, lane 1) and VT2 (3.2 µg, lane 2) into subunits and fragments after SDS-PAGE in reducing buffer. After transfer to nitrocellulose paper, subunits were stained with Colloidal Gold All Protein Stain. Mol.-wt markers (Kda) are shown.

**Fig. 2.** Western-blot analysis of binding of rabbit polyclonal antibodies raised against VT2 to subunits and fragments of VT1 (5 µg, lane 1) and VT2 (3.2 µg, lane 2) resolved after SDS-PAGE in reducing buffer. After electrophoretic transfer to nitrocellulose paper, reaction with anti-rabbit VT2 was followed by incubation with $^{125}$I-labelled goat anti-rabbit IgG and exposure to X-ray film.
Similar results were obtained with MAb 10D11, except that it also reacted weakly with subunit B of VT2. A summary of the characteristics of these MAbs is presented in the table.

Shiga toxin purified from S. dysenteriae serotype 1 was used in ELISA to determine whether MAbs 1C5 (IgG2b; anti-VT2) and 10D12 (IgM; anti-VT1) also recognised Shiga toxin; as expected, both of these MAbs, which can recognise VT1 and VT2, cross-reacted with Shiga toxin.

**Discussion**

Epidemiological evidence suggests that VT1 and VT2 play a role in haemorrhagic colitis and haemolytic uraemic syndrome, the diseases associated with enterohaemorrhagic E. coli (Marques et al., 1986; Scotland et al., 1987). VT1 is biologically, immunochemically and physicochemically identical to Shiga toxin (Noda et al., 1987; Padhye et al., 1989b). Although the biological activity of VT2 is similar to that of VT1, physicochemical analysis has indicated differences in their isoelectric points (Padhye et al., 1987, 1989b; Yutsudo et al., 1987; Downes et al., 1988) and differences in the mol. wts of their A and B subunits. These results, together with the findings that VT1 is antigenically distinct from VT2 and that polyclonal antibodies raised against VT1 do not neutralise biological activity of VT2, indicate that VT2 is physicochemically and immunologically distinct from VT1. However, Jackson et al. (1987) reported an overall deduced amino acid-sequence homology of 55–60% between VT1 and VT2, with localised regions of much higher or lower homology on toxin subunits. Our studies by Western-blot analysis of VT1 and VT2 with MAbs and polyclonal antibodies raised to purified VT1 or VT2 revealed that common epitopes do indeed exist between these toxins, indicating some degree of common sequence homology. The MAbs generated in this study had several interesting properties. For example, despite the use of a prolonged immunisation schedule MAbs to VT1 and VT2 from mice immunised with only heat-treated VT1 or VT2 were predominantly of the IgM class. Similarly, IgM was the predominant class obtained by Perera et al. (1988) in their studies with...
VT2. We had observed before that intraperitoneal injection of VT1 (5-0 μg) was lymphocytotoxic to spleen and intestinal-associated lymphoid tissue (Peyer’s patches, colonic nodules, and mesenteric lymph nodes) of ICR mice (Padhye et al., 1989a). However, similar levels of VT2 did not produce that same response; the effect of these toxins on BALB/c mice is not known. Perhaps the heat-treated toxins, because they are not entirely attenuated, retain some immunosuppressive activity resulting in the predominant IgM response. Interestingly, and in contrast, MAbs produced from mice immunised for a short period with heat-treated and formalin-treated VT1 were principally of the IgG class. Perhaps differences in the degree of attenuation of this toxin preparation, as compared with that of the heat-treated-only toxin preparations, had an influence on the immune response of the mice, thereby affecting the dominant type of immunoglobulin produced.

ELISA results indicated that all MAbs from mice immunised with heat-treated VT1 or VT2 reacted with VT1 as well as VT2. Further characterisation by Western-blot analysis revealed that the MAbs reacted with subunit A and the fragment A₁ of both toxins, but that they did not react with the band of low mol. wt (c. 4–6 Kda) of VT1 or VT2 and which is composed of a mixture of subunit B and fragment A₂. Failure of a particular MAb or various mixtures of MAbs to neutralise biological activity of either of the toxins suggests that all of the MAbs produced from the mice immunised with only heat-treated toxin were produced against an immunodominant epitope on subunit A consisting of a protein domain not responsible for the biological activity of the toxins. However, results do not rule out the possibility that MAbs were reactive against epitopes at different sites on the molecule. Extensive proteolytic digestion and fragmentation of subunit A is required for detailed epitope analyses.

The results obtained with MAb produced from mice immunised with heat-treated and formalin-treated VT1 differed substantially in ELISA procedure, Vero cytotoxicity and mouse-lethality neutralisation assays and Western-blot analyses from those of MAbs from mice immunised with only heat-treated toxin. Two such MAbs (9C9, 3C10) reacted specifically with VT1 and not with VT2 in the ELISA procedure, and with only the subunit B of VT1 and not of VT2 in Western-blot analysis; a third, 10D11, generated against the same VT1 preparation cross-reacted weakly with VT2 in the ELISA procedure and reacted weakly with the B subunit of VT2 in Western-blot analysis. All three MAbs neutralised Vero cytotoxicity and mouse lethality of VT1 but not of VT2, findings suggesting that the B subunit has an important role in determining the specificity and toxicity of VT1 and VT2. Further characterisation of MAbs was restricted by the low recovery of toxins after purification to homogeneity.

We have reported elsewhere (Padhye et al., 1988) that nicking of subunit A of VT2 occurs close to the C-terminus of the molecule because the N-terminal amino-acid sequences of the A₁ fragment and A subunit are identical. MAbs from mice immunised with VT1 or VT2 treated only by heat did not react with the C-terminal A₂ fragment as demonstrated by Western-blot analysis. Again, none of these MAbs reacted with subunit B of either verotoxin. It is likely that denaturation of antigenic determinants on the B subunit might have occurred during the heat treatment of toxin used for immunisations resulting, therefore, in the production of MAbs predominantly against subunit A.

Enterohaemorrhagic E. coli strains are reported to produce VT1, VT2, or both toxins. Whilst assays based on MAbs specific for VT1 or VT2 are useful for detecting individual toxins, these antibodies are of limited use because they recognise only one toxin. MAbs reported in this paper are unique because of their ability to recognise both VTs, and were useful in revealing that VT1 and VT2 share a common epitope(s) comprised of a toxin-domain not responsible for its biological activity, on subunit A. They may also be useful in immunoassays to detect bacteria capable of producing more than one verotoxin.

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