Detection of diphtheria toxin in culture supernates of *Corynebacterium diphtheriae* and *C. ulcerans* by immunoassay with monoclonal antibody

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Summary. Monoclonal antibody (MAb) to diphtheria toxin was produced in mouse hybridomas, and shown by ELISA to be of sub-class IgG₁. Hybridomas were inoculated into mice to produce ascitic fluid from which MAb was purified by caprylic acid. The MAb was shown by immunoblotting to be directed against the A fragment of the toxin and also against the intact toxin molecule. After conjugation with fluorescein isothiocyanate, it was used in an immunoassay to detect toxin in culture supernates of *Corynebacterium diphtheriae* and *C. ulcerans*. The assay correlated well with the Elek test and with virulence in guinea-pigs; but it gave occasional false positive results, probably by binding of MAb to defective toxin.

Introduction

Although diphtheria is now an uncommon disease in the UK, sporadic cases occur which are mainly imported from abroad. Therefore, there is still a need for the clinical laboratory to identify accurately toxin-producing strains of *Corynebacterium diphtheriae*. Toxin tests are traditionally done by the Elek method,¹ with confirmation of virulence in guinea-pigs where animal facilities are available. However, discrepancies amongst laboratories using the Elek test have been reported.² The aim of this study was to produce a monoclonal antibody (MAb) to diphtheria toxin, to investigate its use in an additional laboratory test for toxin production, and to compare this test with existing methods.

Materials and methods

Diphtheria toxin and toxoid

Preparations of toxin and toxoid were supplied by Wellcome Reagents Ltd. The toxin solution contained 5100 Lfu/ml and the toxoid preparation 3400 Lfu/ml.

Immunisation of mice

BALB/c mice were immunised with either diphtheria toxin or toxoid. The protein content of these preparations was estimated by a dye binding assay (Bio-Rad Labora-

tories). Immunisation was by intraperitoneal injection with either 100 µg of toxoid or 50 µg of toxin in Freund's incomplete adjuvant with 50 µg of the muramyl dipeptide (MDP), N-acetylmuramyl L-alanyl D-isoglutamine (Sigma). With toxoid, mice were given three further injections of 10 µg of toxoid in adjuvant with MDP at 2-week intervals. With toxin, they were rested for 4 weeks after the initial injection because of adverse reactions; two injections of 10 µg of toxin were then given, 2 weeks apart. The final inoculation for both groups of mice was 10 µg of toxin or toxoid intravenously, 19 days after the previous injection. Their spleens were removed aseptically 3 days later.

Monoclonal antibody (MAb) to diphtheria toxin

Production. Spleen cells from the immunised mice were fused with mouse myeloma cell line JK-Ag 8653, by standard methods.^{3,4} Culture supernates from hybridomas were screened by an enzyme immunoassay as described below. In the initial screening, 20 hybridoma cultures gave positive test results, and 11 of these were cloned by limiting dilution. Ascitic fluid was obtained with two clones by re-introducing each clone into mice pre-treated with pristane.

Screening of clones for antibody. Wells of a microtitration plate (Nunc, Immunoplate II) were coated with 0.1 ml of a 1 in 1200 dilution of toxin per well, or a 1 in 800 dilution of toxoid, in 0.05 M carbonate buffer (pH 9.6) for 48 h at 4°C. The coated plates were washed three times with phosphate-buffered saline (PBS) containing Tween 20 0.05% v/v (TW). Culture supernates from hybridomas were added and incubated at 37°C for 1 h. Plates were washed as previously and then incubated at 37°C for 1 h

with a 1 in 500 dilution of anti-mouse polyvalent immunoglobulin and alkaline phosphatase conjugate (Sigma). Bound antibody was detected by the addition of phosphatase substrate (Sigma). Plates were examined visually for colour development.

Characterisation and purification. The isotype of the secreted antibody was determined by ELISA. Briefly, antibody was immobilised in wells of a microtitration plate coated with toxin, 0.425 Lfu/well. Bound antibody was then detected with anti-mouse horseradish-peroxidase conjugate specific for IgM (Miles Scientific), IgG whole molecule (Tissue Culture Services Ltd) or IgG subclass (ICN Immunobiologicals), and the appropriate substrate.

MAB was purified from ascitic fluid with caprylic acid.^{5,6}

Immunoblotting (Western blotting)

Samples of toxin and toxoid, treated with sodium dodecyl sulphate (SDS)-mercaptoethanol, were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) in acrylamide gels 10% with a 'Mighty Small' slab gel apparatus (Hoeffer) and the buffer system of Laemmli.⁷ Proteins were transferred to nitrocellulose by electrophoresis.⁸ The nitrocellulose was then incubated for 18 h at 4°C in a solution of bovine serum albumin 5%. The blot was washed in PBS; and a solution of MAB, diluted 1 in 250 in PBS-TW and horse serum (HS) 5%, was layered on to the nitrocellulose and incubated at room temperature for 1 h. The blots were washed as before and alkaline-phosphatase-conjugated anti-mouse IgG (Sigma) was added and incubated for 1 h. After washing, the bound MAB was detected with 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium as the substrate.⁹

Strains of bacteria

Strains of *C. diphtheriae* and *C. ulcerans* were selected from recent isolates sent to the Division of Hospital Infection for investigation, from strains held by the National Collection of Type Cultures (NCTC), Colindale, and from cultures kindly provided by Dr J.M.S. Dixon, Provincial Laboratory of Public Health, Alberta, Canada.

Elek test for toxin production. The toxigenicity of the strains was examined as previously described.¹⁰ Strains NCTC 10648 (rapid toxin producer), NCTC 3984 (weakly toxigenic) and NCTC 10356 (non-toxigenic) were included as controls in each Elek plate.

Virulence in guinea-pigs. One of two guinea-pigs, each weighing c. 250 g, was given 100 units of diphtheria antitoxin (Swiss Serum and Vaccine Institute, Bern) by intraperitoneal injection. A suspension of the test strain was prepared by washing the growth from an 18–24-h Loeffler-slope culture with 2 ml of peptone water; 0.2 ml of this suspension was injected subcutaneously into the protected (control) and unprotected (test) animal. Animals that died were examined *post-mortem* for characteristic effects of diphtheria toxin.

Growth of cultures. Strains were grown in 3 ml of either proteose peptone maltose (PPM) medium¹¹ (Proteose Peptone No. 3 [Difco] 4% w/v, maltose 0.6% w/v), or Elek broth¹² (proteose peptone 2% w/v, maltose 0.3% w/v, NaCl 0.5% w/v, lactic acid 0.07% w/v, NaOH 0.06% w/v). After incubation for 18–24 h at 37°C, cultures were centrifuged to remove the bacteria and the supernates were filtered through a 0.2-µm Acrodisc R filter (Gelman Sciences). Thiomersal 0.02% was added as a preservative and samples were stored at 4°C.

Preparation of MAB labelled with fluorescein isothiocyanate (FITC)

To 2 mg of purified MAB in 0.1 M sodium carbonate and 0.15 M NaCl, 13.7 µl of an ethanolic solution of FITC (5 mg/ml) was added and stirred for 1 h at room temperature. The FITC-conjugated antibody was separated from free FITC by gel filtration on Sephadex G25 in a PD10 column (Pharmacia), and the fractions containing conjugate were pooled. Antibody-FITC conjugates were stored at 4°C in glycerol 20% containing azide 0.08%.

Enzyme immunoassay (EIA) for diphtheria toxin

Microtitration plates (Nunc, Immunoplate II) were coated with 0.1 ml of the purified MAB (5 µg/ml in PBS) for 48 h at 4°C. The plates were washed three times with PBS-TW, and filtered culture supernates (0.1 ml) were added. They were incubated in a moist chamber at 37°C for 1 h, and washed as before. A 1 in 1000 dilution of FITC-conjugated MAB in PBS-TW-HS was added; and they were incubated as before, and washed again. A peroxidase-conjugated anti-FITC MAB⁶ diluted in PBS-TW-HS was added; and they were incubated for 1 h at 37°C, washed, and then incubated for 30 min at room temperature with 100 µl of a peroxidase substrate (42 mM 3, 3', 5, 5'-tetramethylbenzidine and 1.3 mM H₂O₂ in 0.1 M sodium acetate and citric acid buffer, pH 6.0). The reaction was stopped with 0.05 ml of 2 M H₂SO₄, and the optical density (OD) was measured at 450 nm in a Titertek Multiscan MCC/340 MK II (Flow Laboratories).

Results

Two of the hybridoma cultures were stable after two cloning procedures. They were both derived from the same mouse which had been immunised with diphtheria toxoid. The antibody class secreted by both culture supernates was shown by ELISA to be IgG₁. Both clones were inoculated into mice for the production of ascitic fluid. Antibody purified from the fluid by caprylic acid, designated MAB 1 and MAB 2, was used in further studies.

Immunoblotting

Samples of toxin and toxoid, treated with SDS-mercaptoethanol and separated by SDS-PAGE,

were stained with Coomassie Blue (fig. 1). The toxin dissociated into two components which corresponded approximately to the published molecular size of fragment A (21 Kda) and fragment B (37 Kda). The toxoid sample remained largely intact after SDS-mercaptoethanol treatment and was present as a single major band (58 Kda).¹³ Fig. 2 shows a nitrocellulose blot of samples of toxin and toxoid, separated by SDS-PAGE and stained with each MAb. Identical reactions were obtained with both MAbs. Bands were visible in positions on the blot corresponding to the intact toxin molecule and also to the fragment of smaller molecular weight (fragment A) indicating that MAb had bound to these proteins. The blot also showed that there was a distribution of intact molecule and fragment A in both toxoid and toxin samples. The intact molecule predominated in the tracks containing toxoid (1, 3 and 5), whereas toxin was more readily dissociated (tracks 2, 4 and 6).

Fig. 1. Separation of diphtheria toxoid (a) and toxin (b) by SDS-PAGE. Toxoid remained intact, with a single major band (58 Kda); toxin dissociated into two fragments, A (21 Kda) and B (37 Kda).

Activity of purified MAb

Plates were coated with toxin or toxoid, 0.425 Lfu/well. Doubling dilutions of each purified MAb, from 1 in 500, were added. Bound antibody was detected with anti-mouse polyvalent immunoglobulins conjugated to alkaline phosphatase. A consistently stronger signal was obtained with toxin than with toxoid, although both MAbs were raised against the toxoid.

Detection of toxin in culture supernates by EIA

FITC conjugates were prepared from purified MAbs 1 and 2. Preliminary experiments were done to determine the effect of using either the same MAb as both capture and detecting antibody, or one MAb to capture and the other to detect. Wells of a microtitration plate were coated with either MAb 1 or 2. Serial dilutions of toxin were added, and also either FITC-conjugated MAb 1 or 2. When MAb 1 was used for both capture and detection, the EIA result was negative. Coating with MAb 1 and detecting with MAb 2 (MAb 1/2) resulted in lower OD readings than coating with MAb 2 and detecting with MAb 1. The latter was roughly equivalent to coating and detecting with MAb 2.

With both the MAb 1/2 and MAb 2/1 sequences, toxin could be detected at a dilution of 5×10^{-5} Lfu/ml. In initial experiments with culture supernates, tests were performed in duplicate, with MAb 1/2 and MAb 2/1 sequences. Later, when all the culture supernates were tested in the same experiment (fig. 3), the sequence MAb 2/1 was used.

A total of 78 strains, including four toxigenic strains of *C. ulcerans*, was tested by EIA for toxin activity. The results were very clear, and the strains could be divided into two distinct groups by visual examination of the plate for the presence of colour in each well. Measurement of the OD at 450 nm confirmed that there was a significant difference between the EIA positive (tox⁺) and the EIA negative (tox⁻) supernates (fig. 3). For all except two of the toxin-positive strains, the OD was > 1.0 when grown in both media. When grown in Elek broth, 32 (78%) of 41 positive strains gave OD values > 1.5, compared with only 21 of the same strains (51%) grown in PPM medium. In contrast, all toxin-negative strains, except one grown in PPM medium, gave OD values < 0.1, usually < 0.05.

Five strains from the NCTC were tested. Two of these gave consistently low values with both media. Three control strains for the Elek test gave the expected results in the EIA test, two positive and

Fig. 2. Nitrocellulose blot of diphtheria toxoid and toxin, separated by SDS-PAGE: toxoid in tracks 1, 3 and 5, toxin in tracks 2, 4 and 6; tracks 1 and 2 stained with MAb 1, tracks 3–6 with MAb 2.

one negative, but there was no significant difference in OD between the strongly toxigenic (NCTC 10648) and the weakly toxigenic (NCTC 3984) strains. The OD values of these three strains were similar to those of recent isolates.

Influence of growth medium on EIA values

Of the 41 strains that were positive by EIA, the OD with Elek broth was higher than with PPM medium for 32 strains. PPM cultures gave higher

readings in five cases, and there was no appreciable difference with four strains.

Comparison of EIA with other methods of toxin detection

All strains tested by EIA were tested also by the Elek method (see table); and 18 were inoculated into guinea-pigs for the virulence test, including all with which there was a discrepancy between the EIA and the Elek results.

Fig. 3. Detection of diphtheria toxin by EIA. Whether cultures were grown in Elek broth (fig. 3A) or PPM medium (fig. 3B), there was a clear difference between EIA-positive (●) and EIA-negative strains (○), though in general the positive strains gave higher readings from Elek broth (see text).

There was agreement between the EIA and Elek results with 74 of the 78 strains tested. Of these, 14 were also injected into guinea pigs, and again there was agreement. There were discrepancies with four strains, all from the Canadian collection. All four strains were EIA positive. This was confirmed by repeated testing with the same culture supernates and also with a fresh culture of each strain. Two gave equivocal results in our Elek test which were difficult to interpret but were most probably weakly positive. However, only one was virulent in a

guinea-pig, causing death within 48 h. Animals inoculated with each of the other three remained well for 14 days after injection.

Discussion

Diphtheria is now a rare disease in many parts of the world. During 1980–1986 only 27 cases were reported in England and Wales,¹⁴ mainly imported from abroad. However, outbreaks may still occur among susceptible individuals, as illustrated in a group of alcoholics in Sweden¹⁵ with 13 clinical cases and 65 carriers. Despite high levels of immunisation in children, serological surveys in Sweden have shown that 19% of people under 20 years and 68% over 60 years had antibody below the protective level of 0.01 IU/ml.¹⁶ In similar studies in Britain, 35% of the population were susceptible.¹⁷ Therefore, the need for laboratory identification of toxigenic strains of *C. diphtheriae* still exists.

Possibly even more emphasis is now placed on bacteriological studies because diphtheria is often not considered in the initial diagnosis.¹⁸ The carriage of non-toxigenic strains is not uncommon.¹⁹ Therefore, it is important to identify toxin-producing strains accurately in the laboratory, primarily to confirm the diagnosis of diphtheria but also to contain the possible spread of the disease by tracing contacts who may be carriers.

Toxigenicity is usually assessed by the Elek test with hyperimmune horse antitoxin. Non-specific precipitation lines may occur and lead to confusion, particularly if the plate is incubated for 48 h or

Table. Comparison of enzyme immunoassay (EIA), the Elek test and guinea-pig inoculation, for the detection of toxin production by strains of *C. diphtheriae*

Number of strains	Detection of toxin by		
	EIA	Elek test	Virulence in guinea-pigs
5	—	—	—
32	—	—	NT
28*	+	+	NT
9*	+	+	+
1†	+	—	—
1‡	+	—	—
1†	+	(+)	—
1†	+	(+)	+

NT, Not tested.

(+), Weak positive.

* Includes two strains of *C. ulcerans*.

† Designated as tox⁺ by the referring laboratory.

‡ Designated as tox[−] by the referring laboratory.

longer.¹ Also the amount of toxin produced by a strain depends on the concentration of iron in the medium²⁰ and, therefore, if the test conditions are not optimal, erroneous results may be reported. Because the Elek test is performed infrequently in most laboratories, this is a serious problem.² Confirmatory tests are therefore required. In the present study, MAbs have been used in an immunoassay to detect toxin in culture supernates.

A comparison of PPM medium and Elek broth was made because both media had been used previously in other studies of toxin production. Although the OD₄₅₀ was higher for the majority of strains with Elek broth, the division into EIA-positive and EIA-negative strains was the same irrespective of the growth medium.

Our results showed that there was agreement between the Elek test and EIA for 96% of strains tested. Identical results were obtained when 18% of these isolates were tested for virulence in guinea-pigs. With one strain, EIA confirmed a weak positive Elek result which was also substantiated by a positive guinea-pig test. The EIA appeared to give false positive results with three isolates on the basis of negative guinea-pig tests. Two of these strains were also negative in our Elek test although one had been designated as a toxin producer by the referring laboratory. Since both of our MAbs were directed against the A fragment of diphtheria toxin, it is unlikely that a strain that gave a positive reaction in the EIA would fail to produce toxin. However, it is possible that a strain producing a defective, inactive toxin might give a positive EIA but be avirulent in a guinea-pig. In such a case, a positive result might be expected also in the Elek test.

It is an advantage if a test for toxin production provides quick results. Antibody-coated plates were

stable for several weeks after storage at 4°C, and are, therefore, ready for immediate use. Cultures are grown for 18 h, and the EIA test takes 5 h to perform. Therefore the EIA results are available within 2 days of receiving the culture.

The EIA could detect diphtheria toxin at a concentration of $c. 5 \times 10^{-5}$ Lfu/ml, or 88 pg/ml. In other reports, the sensitivity has varied widely: 10^{-6} Lfu/ml,²¹ 10 ng of toxin/ml (5.7×10^{-3} Lfu/ml),²² 0.6 ng/ml (3.4×10^{-4} Lfu/ml),¹¹ assuming that 1 Lfu is equivalent to 1.75 µg of toxin protein.²³

Nielsen *et al.*²² described a double antibody sandwich ELISA in which horse antitoxin was used as the capture antibody and MAb for detection. Bacteria were grown directly in the ELISA plate, which presented a safety hazard; to minimise this, ethanol was added to the wells before the bacteria were washed out. This may explain the low OD values for their toxigenic strains. We have used filtered culture supernates, with no safety hazard; and the average OD for toxigenic strains grown in Elek broth was 1.65.

In immunoblotting experiments, both of our MAbs bound to intact toxin and to fragment A. By comparison, Hayakawa *et al.*²⁴ studied 14 MAbs produced by immunisation with toxoid, and only two of these bound to fragment A. Our preliminary EIA experiments suggested that MAb 1 and MAb 2 recognised different epitopes, and that MAb 1 may be directed against an epitope which is present or accessible only in low concentration.

This study has shown that EIA provides an additional confirmatory test for diphtheria toxin which should be useful in a reference laboratory. Although it gives occasional false-positive results, it does not appear to give the equivocal results sometimes found with the Elek test.

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