Development of a monoclonal antibody binding okadaic acid and dinophysistoxins-1, -2 in proportion to their toxicity equivalence factors

Stewart, L., Elliott, C., Walker, A., Curran, R., & Connolly, L. (2009). Development of a monoclonal antibody binding okadaic acid and dinophysistoxins-1, -2 in proportion to their toxicity equivalence factors. Toxicon : official journal of the International Society on Toxinology, 54(4), 491-498. DOI: 10.1016/j.toxicon.2009.05.015

Published in:
Toxicon : official journal of the International Society on Toxinology

Toxicon xxx (2009) 1–8

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Development of a monoclonal antibody binding okadaic acid and dinophysistoxins-1, -2 in proportion to their toxicity equivalence factors

Linda D. Stewart [a,*], Christopher T. Elliott [a], Andrew D. Walker [b], Rhonda M. Curran [a], Lisa Connolly [a]

[a] Institute of Agri-food and Land Use, School of Biological Sciences, Queen's University Belfast, David Keir Building, Stranmillis Road, Belfast, BT9 5AG, Northern Ireland, UK
[b] Xenosense Ltd., Unit 18, The Innovation Centre, Titanic Quarter, Queen's Road, Queen's Island, Belfast BT3 9DT, Northern Ireland, UK

ARTICLE INFO

Article history:
Received 3 February 2009
Received in revised form 18 May 2009
Accepted 19 May 2009
Available online xxxx

Keywords:
Okadaic acid
Monoclonal antibodies
Fusion screening
Biosensor
Toxicity equivalence factors

ABSTRACT

Okadaic acid (OA) and structurally related toxins dinophysistoxin-1 (DTX-1), and DTX-2, are lipophilic marine biotoxins. The current reference method for the analysis of these toxins is the mouse bioassay (MBA). This method is under increasing criticism both from an ethical point of view and because of its limited sensitivity and specificity. Alternative replacement methods must be rapid, robust, cost effective, specific and sensitive. Although published immuno-based detection techniques have good sensitivities, they are restricted in their use because of their inability to: (i) detect all of the OA toxins that contribute to contamination; and (ii) factor in the relative toxicities of each contaminant. Monoclonal antibodies (MAbs) were produced to OA and an automated biosensor screening assay developed and compared with ELISA techniques. The screening assay was designed to increase the probability of identifying a MAb capable of detecting all OA toxins. The result was the generation of a unique MAb which not only cross-reacted with both DTX-1 and DTX-2 but had a cross-reactivity profile in buffer that reflected exactly the intrinsic toxic potency of the OA group of toxins. Preliminary matrix studies reflected these results. This antibody is an excellent candidate for the development of a range of functional immunochemical-based detection assays for this group of toxins.

1. Introduction

A number of marine phytoplankton, including several species of dinoflagellates and diatoms, produce biotoxins, which accumulate and become concentrated by shellfish. Consumption of shellfish sufficiently contaminated by such toxins can result in human illness. The intermittent and random outbreaks of these biotoxins are a major threat to the global shellfish industry but this risk, however, is managed by monitoring programmes where rapid and early detection of toxins is crucial.

The diarrhetic shellfish poisoning (DSP) toxins are amongst the most widely occurring toxin groups in shellfish worldwide and include OA and its analogue DTX-1, isomer, DTX-2 and ester DTX-3. These are a group of thermostable, polyether and lipophilic compounds causing a gastrointestinal illness with the predominant symptoms of diarrhoea, nausea, vomiting and abdominal pain (Aune et al., 2007; Jorgensen et al., 2005). DSP incidents have been reported worldwide with OA outbreaks more frequently associated with Europe (Kreuzer et al., 1999) while in Japan DTX-1 is more prevalent (Hallegraeff, 1995) and in Ireland, the principal DSP toxin detected is DTX-2 (Carmody et al., 1996). DTX-3 toxic episodes have been reported in Norway, Chile, Portugal and Spain (Vale and Sampayo,

* Corresponding author. Tel.: +44 2890976562; fax: +44 2890976513.
E-mail address: l.stewart@qub.ac.uk (L.D. Stewart).

0041-0101/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2009.05.015

Please cite this article in press as: Stewart, L.D., et al., Development of a monoclonal antibody binding okadaic acid and dinophysistoxins-1, -2 in proportion to their toxicity equivalence factors, Toxicon (2009), doi:10.1016/j.toxicon.2009.05.015

2002; Torgersen et al., 2005; Garcia et al., 2005; Villar-Gonzalez et al., 2007). Single toxic episodes can be a complex phenomenon involving several distinct groups of natural compounds with the predominant toxin varying in different toxic events.

The current reference method under EU legislation (EC, 2002) and the most widely used method for the analysis and control of these toxins is the MBA (Yasumoto et al., 1978). This method is under increasing criticism not only from an ethical point of view but also because of its limited sensitivity and inability to distinguish between toxins of different groups (Quilliam and Wright, 1995; Hess et al., 2006). A number of alternative methods have been proposed ranging from cytotoxicity assays (Aune et al., 1991; Amzil et al., 1992.; Tubaro et al., 1996a), protein phosphatase assays (Luu et al., 1993; Honkanen et al., 1996; Simon and Vernoux, 1994; Tubaro et al., 1996b; Isobe et al., 1995; Vieytes et al., 1997), liquid chromatography (LC) coupled with fluorescence detection and LC coupled with mass spectrometry, capillary electrophoresis and numerous immunosorbent methods (James et al., 2000). Some of these methods lack the required sensitivity and others require long sample preparation. Antibody-based immunoassay procedures offer the opportunity of high throughput screening of samples. There are immuno-based test kits commercially available for the detection of OA, including ERFA biotech, DSP-check (Sceti, Japan) and E.F.2 (CER, Belgium), however, some require the coating of plates with OA making the kits very expensive, and only the E.F.2 kit provides limited cross-reactivity data (McNabb, 2008). Pure shellfish toxins are extremely difficult and expensive to source so the amount of toxin used in an assay system is an important consideration. If an analytical-based detection method is to be used to replace the MBA it would be desirable that it should not only identify all toxic analogues that contribute to contamination but also be able to factor in their relative toxicities. The toxicological database for OA group toxins is limited and comprises mainly studies on their acute toxicity. To facilitate the replacement of MBA, toxicity equivalence factors (TEFs) for each of the analogues OA, DTX-1 and DTX-2 have been established as 1, 1, and 0.6 respectively based on LD_{50} experiments following intraperitoneal injection in mice and PP2A inhibition assays (Aune et al., 2007). This has resulted in issues relating to the ability to detect the OA toxin and its analogues relative to their differing TEFs in analytical screening methods. The ideal bio-tool of the future should therefore have low detection limits, be able to deal with complicated matrices and toxin mixtures, use minimal amount of toxin, should have a high sample throughput capability to manage increased testing, and the ability to quantify the total amount of toxin based on differing TEFs.

Immuno-based biosensors, using the principle of surface plasmon resonance (SPR), are a highly promising bio-tool for screening. They produce rapid and reliable results with minimal sample preparation and use of analyte (Elliott et al., 1999; Gaudin et al., 2001; Gustavsson et al., 2002; Haasnoot et al., 2002; Mello and Kubota, 2002; Samsonova et al., 2002; Ferguson et al., 2002, 2005; Gaudin et al., 2005; Traynor et al., 2006; Mauriz et al., 2006; Llamas et al., 2007; Campbell et al., 2007; Connolly et al., 2007, Campas et al., 2007). This technology allows for real time, automated analysis combining the high affinity of biochemical interactions with low limits of detection and is characterised by its simplicity of use. The production of antibodies is of fundamental importance to develop immunobiosensor assays but the highly toxic potencies and low molecular weights of algal toxins complicate procedures for antibody production. An SPR biosensor assay was previously developed for the detection of OA from shellfish matrices using a polyclonal antibody (Llamas et al., 2007). While the assay was proven to be sensitive and robust, the specificity of the antibody used was an issue in that it failed to detect DTX-1 in matrix.

Monoclonal antibodies are an alternative binder for use in biosensor assays. They are proteins which, if carefully selected, can exhibit excellent sensitivity and specificity in their interactions with specific sites on target molecules. The basis of antibody detection is structural recognition and not toxicity so in many cases the ultimate usefulness of a MAb will lie in its specificity and cross-reactivity profile. During MAb production hundreds of potential antibodies are produced and fast, confident decisions must be made as to which hybridomas should be cloned. Candidate antibodies are assessed during screening for their ability to bind the target antigen, in the present example, a MAb produced to OA should also have the capability to bind to the important OA toxins, DTX-1 and DTX-2. The rapid detection of ligand binding activity and further characterisation of antibody binding properties are crucial in the accurate prediction of their performance. The early identification of MAbs with the desired affinity and specificities are the most critical steps during the selection process and so the intended application of the MAbs is an important consideration when choosing the appropriate screening strategy (Zilka et al., 2003).

In the present study hybridoma technology was used to attempt to generate MAbs against OA with significant binding to the DTX toxins. Traditionally ELISA assays are used to screen the products of a MAb fusion, however, as the intended end method for the MAb was a biosensor assay, an automated biosensor-based screening assay was designed and developed for the hybridoma selection process and compared with commonly used ELISA techniques. The production and screening of MAbs specific for the OA group of toxins and the subsequent identification and characterisation of an exceptional MAb to OA is described.

2. Materials and methods

2.1. Source of reagents and apparatus

An optical SPR biosensor system (BIACORE Q), BIACORE Q control and evaluation software, CM5 sensor chip (research grade), HBS-EP buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% polysorbate 20 (v/v), pH 7.4) and 1 M ethanolamine hydrochloride were supplied from Biacore AB (Uppsala, Sweden). Bovine thyroglobulin (BTG), bovine serum albumin (BSA), cationised-bovine serum albumin (cBSA), horseradish peroxidase (HRP), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride

Please cite this article in press as: Stewart, L.D., et al., Development of a monoclonal antibody binding okadaic acid and dinophysistoxins-1, -2 in proportion to their toxicity equivalence factors, Toxicon (2009), doi:10.1016/j.toxicon.2009.05.015

(EDC) and *N*-hydroxysuccinimide (NHS) and all organic solvents (Analar grade) were obtained from Sigma–Aldrich Chemical Company (Dorset, UK). The Quil A was from Superfos and the $Pam_3Cys\text{-}Ser(Lys)_4\text{-}OH$ (PCSL) from EMC Microcollections, GmbH, (Germany). Isotyping kits were obtained from Roche (IsoStrip) and the anti-mouse IgG microtitration strips from Perkin Elmer (Finland). The Nunc Maxisorp plates were bought from Rosklide (Denmark) and the horseradish peroxidase-linked anti-mouse immunoglobulin from DAKO (Cambridge, UK). TMB/E solution was obtained from Chemicon International. OA was acquired from LC Laboratories (USA) and the OA standard was purchased from NRC, Canada. DTX-1 standard was a gift from Biosense Laboratories (Norway) and DTX-2 standard was a gift from the Marine Institute, Galway (Ireland).

2.2. Preparation of okadaic acid-BTG (OA-BTG) immunogen

The OA-BTG immunogen was prepared as described by Llamas et al. (2007).

EDC and NHS were dissolved in MES buffer and added to a solution containing OA in dimethylsulphoxide (DMSO). After activation of this mixture, BTG dissolved in phosphate buffered saline was added along with pyridine. After 12 h incubation at 25 °C it was purified by dialysis against saline solution.

2.3. Immunisation regime and fusion

BALB/c mice were immunised at 3-week intervals with 20 μg of OA-BTG immunogen. A primary and secondary booster immunisation was administered using Quil A adjuvant by subcutaneous injection. A third booster was administered similarly except with PCSL adjuvant by intraperitoneal injection. The fourth booster was administered with 80 μg of immunogen in PCSL adjuvant by intraperitoneal injection. The mice were monitored by ELISA and biosensor for specific antibody titre using tail bleeds taken 10 days after each booster. The most responsive mouse was chosen and 4 days prior to the fusion being performed, received a final booster intraperitoneally of 100 μg of immunogen in phosphate buffered saline (PBS) (pH 7.2) only. A single cell suspension was prepared from the spleen of the immunised mouse and fused with SP2 cells using polyethylene glycol according to a modification of the method of Kohler and Milstein (1975). Serum from the final heart bleed of the fusion mouse was used as a positive control in the screening assays.

2.4. Screening procedures

Optimisation of the screening procedures was carried out on the final heart bleed of the fusion mouse.

2.4.1. ELISA screening procedure I: competitive assay

2.4.1.1. Preparation of OA-HRP conjugate. OA (0.5 mg) was dissolved in DMSO (100 μl) and 10 mM sodium acetate (NaOAC) pH 4.5 (75 μl) buffer added. EDC (3 mg) was dissolved in 10 mM NaOAC (pH 4.5) (50 μl) buffer and NHS (1.5 mg) was dissolved in 10 mM NaOAC (pH 4.5) (50 μl) buffer. EDC solution and NHS solution (50 μl, 1:1; v/v) were added to the OA acid solution and the mixture stirred at ambient temperature for 15 min. The mixture was then added to a solution of HRP (2.5 mg) in PBS pH 7.2 (250 μl) and stirred overnight at ambient temperature. The OA-HRP protein conjugate was then purified by dialysis in saline solution (9 g NaCl/l) with the saline solution changed 3 times.

2.4.1.2. Competitive ELISA protocol. Hybridoma supernatant was diluted 1:4 in 1 mM NaOAc buffer (pH 7.2) and 100 μl of this added to four wells of anti-mouse IgG microtitration strips. The positive control was the final heart bleed of the mouse and the negative control was cell culture medium both diluted in 1 mM NaOAc buffer (pH 7.2). The plate was sealed and incubated at room temperature overnight. The following day the supernatant was discarded and 1/4000 OA-HRP (50 μl) made up in 2 mg/ml BSA in 1 mM NaOAc buffer (pH 7.2) buffer was added to each well. OA standard (50 μl, 10 ng/50 μl) was added to two of the four wells and 1 mM NaOAc buffer (pH 7.2) (50 μl) to the other two wells. The plate was incubated for 3 h at 37 °C in an incubated shaker and then washed five times using wash buffer (NaCl 9 g/l, 1.25 ml 10% Tween 20). TMB/E (100 μl) solution was added to each well and 12 min later the reaction was stopped by adding 2.5 M sulphuric acid (H_2SO_4) (25 μl). The plate was read on a spectrophotometer at 450 nm. A result was considered positive if the reading of the wells containing no OA was greater than 1.0 and the reading in the two corresponding wells containing OA was less.

2.4.2. ELISA screening procedure 2: antigen coated assay

2.4.2.1. Preparation of OA-cBSA conjugate. The OA-cBSA conjugate was prepared using the same protocol as for the OA-HRP except the EDC/NHS/OA solution was added to a solution of cBSA (2 mg) in PBS buffer (pH 7.2) (250 μl) and stirred overnight at ambient temperature. The OA-cBSA protein conjugate was then purified by dialysis in saline solution (9 g NaCl/;) with the saline solution changed 3 times. The conjugate was freeze-dried and reconstituted in deionised water to a concentration of 1 mg/ml.

2.4.2.2. Antigen coated ELISA protocol. Nunc Maxisorp plates were coated with OA-cBSA conjugate (1 μg/100 μl/per well in 0.1 M bicarbonate buffer pH 9.4–9.7) and incubated at 4 °C overnight. The conjugate was discarded and the plates blocked using 1% gelatine in phosphate buffer (PB) pH 7.2 (200 μl/well) and incubated overnight at 4 °C. The blocking buffer was discarded and hybridoma supernatant (100 μl) diluted 1:4 in PB buffer (pH 7.2) added to two wells of the plate. The positive control was the final heart bleed of the mouse and the negative control was cell culture medium diluted in PB (pH 7.2). The plates were incubated in an incubator shaker for 1.5 h at 37 °C, the solution discarded and the plate washed three times using wash buffer. Secondary antibody (horseradish peroxidase-linked anti-mouse immunoglobulin) (100 μl) was added to each well and incubated at 37 °C for 1 h. The secondary antibody was

Please cite this article in press as: Stewart, L.D., et al., Development of a monoclonal antibody binding okadaic acid and dinophysistoxins-1, -2 in proportion to their toxicity equivalence factors, Toxicon (2009), doi:10.1016/j.toxicon.2009.05.015

discarded and the plates washed three times with wash buffer. TMB/E (100 μl) was added to each well and the reaction stopped after a few minutes using 2.5 M H_2SO_4 (25 μl). The plates were read at 450 nm using a spectrophotometer. A result was considered positive if the reading was greater than 1.0.

2.4.3. Biosensor screening assay

2.4.3.1. OA immobilisation onto a CM5 sensor chip surface. The method described previously (Llamas et al., 2007) was used to immobilise the OA onto the surface of a CM5 chip surface. Briefly, the carboxymethylated surface of a CM5 sensor chip was equilibrated to room temperature and an EDC/NHS mixture (50 μl, 1:1; v/v) was incubated on the chip surface for 30 min. The excess solution was removed and freshly prepared 1 M ethylene diamine (pH 8.5) (50 μl) was added to the NHS-ester chip surface and incubated for 1 h. The surface of the chip was washed with HBS-EP buffer and the free NHS-ester groups were blocked with 1 M ethanolamine hydrochloride (pH 8.5) (50 μl) for 20 min. EDC (54.0 mg) and NHS (24.0 mg) were dissolved in 10 mM NaOAc buffer (pH 4.5) (1.0 ml). An aliquot of this mixture (10 μl) was added to OA (50 μg), which was previously dissolved in DMSO (10 μl) and 10 mM NaOAc buffer (pH 4.5) (30 μl). OA-NHS derivative (50 μl) was then added to the amine chip surface and incubated for 4 h. The excess solution was removed and the chip surface was washed with deionised water, dried under a gentle stream of nitrogen gas and stored desiccated (4 °C) when not in use.

2.4.3.2. Biosensor protocol. Hybridoma supernatant diluted 1:1 in HBS-EP buffer was analysed using a concentration analysis wizard on the Biacore Q system (flow rate 25 μl/min, volume 25 μl). The chip surface was regenerated using 15% acetonitrile in 180 mM sodium hydroxide (NaOH) (flow rate 25 μl/min, volume 25 μl). A result was considered positive if the response was greater than 100 response units (RU). For antibody characterisation studies, standard solutions of OA and DTX-1 (50 ng/ml) in HBS-EP buffer were made from a stock standard of each toxin (10 μg/ml OA/DTX-1 in MeOH). For the standard calibration curves in buffer, working standards (100, 50, 20, 10, 5 and 1 ng/ml) were made by dilutions in HBS-EP buffer from stock standard solutions of OA, DTX-1 and DTX-2 in methanol.

2.5. Isotyping of antibodies

The resulting antibodies were isotyped according to the instructions for the isotype kit (IsoStrip).

2.6. Preliminary matrix studies

2.6.1. Sample extraction procedure

A 1 g portion of known negative mussel tissue homogenates (i.e. from shellfish tested negative for DSP by MBA) was weighed into glass bottles. Aqueous acetonitrile (90%) (10 ml) was added and the sample vortexed for 5 s prior to roller mixing (30 min). After centrifugation (3500 rpm, 10 min at 10 °C) an aliquot of each supernatant (1 ml) was transferred into a glass tube and evaporated to dryness (45 °C) under a gentle stream of nitrogen. The residue was reconstituted in HBS-EP (950 μl) and appropriate standard concentration (50 μl) added. Working standards (250, 200, 150, 100, 50 ng/g) for matrix calibration curves were made by dilutions in HBS-EP buffer from stock standard solutions of OA, DTX-1 and DTX-2 in methanol. The samples were vortexed for 20 s and filtered through a 0.45 μm Millex-HA filter prior to analysis.

2.6.2. Biosensor protocol: standard calibration curve in matrix

The diluted shellfish extracts were mixed 1:1 with OA MAb. Each sample (50 μl) was injected over the sensor chip surface at a flow rate of 25 μl/min. Report points were recorded before and after each injection and the chip surface was regenerated with 25 μl injection (flow rate 25 μl/min) of 30% acetonitrile in 220 mM NaOH.

3. Results and discussion

Between 10 and 14 days post fusion, 415 hybridoma supernatants were assayed by the three screening methods (direct ELISA, indirect ELISA and biosensor analysis). All positive hybridomas, i.e. those showing specific binding to OA, were subsequently isotyped using a mouse monoclonal isotyping kit (Table 1).

The direct ELISA screening of the 415 hybridoma supernatants revealed 16 positive reactors. Testing of the same samples by indirect ELISA resulted in 18 being deemed as positive binders to OA. Two of these were later classified as false positives as competition analysis, introducing OA into the indirect ELISA format, determined that the binding was non-specific. These two hybridomas were also found to be negative by both biosensor and direct ELISA. Both the direct and indirect ELISA procedures failed to detect one positive (hybridoma no. 13). The biosensor assay detected 17 positives, all of which were later confirmed to be true OA binding antibodies, i.e. all showed

Table 1
Screening assay results from the three techniques employed and the isotypes of the antibodies detected (+, detected; –, not detected).

Hybridoma no.	Isotype	Biosensor	Antigen coated ELISA	Competitive ELISA
1	IgG2a K	+	+	+
2	IgG1 K	+	+	+
3	IgG1 K	+	+	+
4	IgG2a K	+	+	+
5	IgG1 K	+	+	+
6	IgG1 K	+	+	+
7	IgG1 K	+	+	+
8	IgG1 K	+	+	+
9	IgG2a K	+	+	+
10	IgG1 K	+	+	+
11	IgG2a K	+	+	+
12	IgG2a K	+	+	+
13	IgG1 K	+	–	–
14	IgG2a K	+	+	+
15	IgG1 K	+	+	+
16	IgG1 K	–	+	–
17	IgM K	–	+	–
18	IgG1 K	+	+	+
19	IgG2a K	+	+	+

Please cite this article in press as: Stewart, L.D., et al., Development of a monoclonal antibody binding okadaic acid and dinophysistoxins-1, -2 in proportion to their toxicity equivalence factors, Toxicon (2009), doi:10.1016/j.toxicon.2009.05.015

inhibition of binding in the presence of OA in subsequent analysis. There was no evidence of this procedure failing to detect any anti-OA secreting hybridomas. The results also revealed that this screening procedure did not detect any false positive results which are tedious and time consuming to deal with. This may be due to a combination of the very short contact period between the solid phase and the supernatants and the continuous flow system employed, both of which serve to reduce non-specific binding.

The existence of a positive hybridoma (no. 13) specific to the measurement method suggests that this positive may depend on the principle of the screening method rather than on a measurement error. ELISA assay procedures generally detect only high affinity antibodies with slow off rates as they involve a number of washing steps which, as well as being time consuming, introduce the possibility of dissociation of low affinity, high off rate antibodies from the detecting molecules. In contrast, the biosensor-based screening permits the detection of a broad range of antibody/antigen interactions including low affinity binding. However, the sensorgram produced for hybridoma 13 (Fig. 1) indicated that the interactions displayed between the immobilised toxin and antibody had a slow off rate and an antibody of potential use in biosensor, ELISA and lateral flow-based procedures had been identified. Hybridoma no. 13 was subsequently found to be slow growing and was likely to have been producing insufficient specific antibody at the time of screening for ELISA-based detection. The high sensitivity of the biosensor procedure most probably contributed to its recognition by this technique. When retested by both ELISA assays several days later, hybridoma no. 13 was found to be positive.

The sensorgrams generated for each interaction are produced in real time and can convey a range of other useful information relating to the interaction characteristics such as affinity and ability to be dissociated from the immobilised ligand by the chip regeneration solution utilised. Sensorgrams allow monitoring of not only the binding event itself but each step in the process of association and disassociation and provides quality control of the entire assay procedure. In contrast the ELISA formats used provide a single end point result for each hybridoma tested.

In a number of antibody production applications and in particular screening for antibodies to toxins a substantial technical difficultly lies in the availability of sufficient quantities of the toxin. In the present study, the direct ELISA assay format, required 500 μg to produce the OA-HRP conjugate. The indirect ELISA assay format also required 500 μg of OA toxin to produce the OA-cBSA conjugate. In the biosensor assay 50 μg of toxin was used to produce the chip surface employed throughout the entire screening procedure.

The evaluation of all the data produced by the three screening tests showed that the indirect ELISA produced both false positive and false negative results, the direct ELISA produced false negative results and, with no false positive or false negative results, screening by biosensor was considered to be the most reliable in selecting the positive clones. Also biosensor screening was the most efficient method in relation to toxin usage, could be the most suitable method for determining differential cross-reactivity profile for OA, DTX-1 and DTX-2, and was the intended end method, so the decision was taken to screen the first and second cloning stages of the fusion by this method only.

The ultimate usefulness of the MAb will lie in its specificity and cross-reactivity profile. A MAb produced to OA should have the capability to bind to the important OA analogues, DTX-1 and DTX-2. A further complication lies in the fact that for the OA group of toxins the relative toxicity factors for OA, DTX-1 and DTX-2 have been established as 1, 1, and 0.6 respectively (Aune et al., 2007). Therefore the 'perfect MAb' for these toxins would not only bind each of them but also reflect their differential toxicities. To increase the possibility of identifying a MAb that cross-reacted with DTX-1, inhibition testing of the selected hybridomas, comparing the differential binding efficiency of each in the presence of 50 ng/ml standards of OA and DTX-1, was introduced. Limited availability of DTX-2 dictated that it could not be included at this stage of the screening process. Each clone was screened by biosensor using 1:1 ratio hybridoma supernatant to HBS-EP buffer to determine binding to the chip surface, 1:1 ratio hybridoma supernatant to 50 ng/ml OA to determine if the antibody was specific for OA and 1:1 ratio hybridoma supernatant to

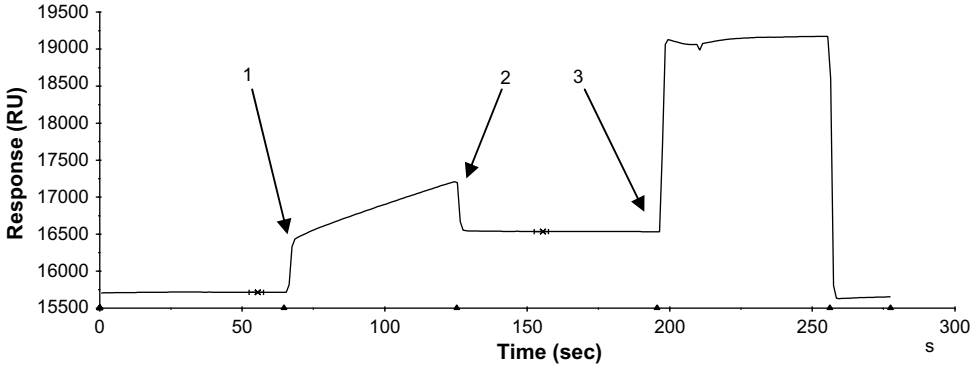

Fig. 1. Biosensor sensorgram for hybridoma no. 13. The shape of the sensorgram indicated that the antibody had a slow off rate (i.e. low dissociation observed) and was a suitable for many forms of immunoassays. Phase 1, association of antibody with toxin on chip surface; phase 2, dissociation of antibody from the chip surface; phase 3, addition of surface regeneration solution.

Please cite this article in press as: Stewart, L.D., et al., Development of a monoclonal antibody binding okadaic acid and dinophysistoxins-1, -2 in proportion to their toxicity equivalence factors, Toxicon (2009), doi:10.1016/j.toxicon.2009.05.015

Table 2
Characterisation of monoclonal antibodies using the biosensor procedure.

Monoclonal antibody	% inhibition using 50 ng/ml standards	
	OA	DTX-1
QUB-OA-1	96	95
QUB-OA-2	94	97
QUB-OA-3	96	96
QUB-OA-4	96	90
QUB-OA-5	98	97
QUB-OA-6	98	96
QUB-OA-7	98	98

50 ng/ml DTX-1 to determine cross-reactivity with DTX-1. This method allowed the selection of clones capable of binding both OA and DTX-1 and with the greatest degree of sensitivity as judged by the degree of binding inhibition observed by toxin presence. The percentage inhibition to both OA and DTX-1 was calculated as 100 − (OA 50 ng/ml response (RU)/negative response (RU) × 100) = % inhibition. The characterisation studies indicated that from the original 17 hybridoma cell lines seven candidates showed >90% inhibition of binding in the presence of the two toxins and were easily removed from the chip surface during the regeneration process (Table 2). These cell lines were selected for additional characterisation as potential binders to be employed in an SPR-based assay. An additional advantage of screening by the intended end method was evident at this stage as several clones were identified which, although they produced high affinity antibodies, were difficult to remove from the chip surface during the regeneration process. These clones were eliminated at this selection stage but not discarded as they may prove extremely useful in other immunoassay formats such as lateral flow or ELISA.

Further characterisation studies involved the production of buffer calibration curves for each of the seven chosen MAbs for OA (Table 3). From these results the two MAbs with the lowest calibration curve midpoints (IC_{50}s) for OA (QUB-OA-1 and OA-7) were selected and the degree of cross-reactivity against DTX-1 and DTX-2 determined by producing standard calibration curves in buffer with each toxin and determining the midpoints of each curve (Table 4). Cross-reactivity was calculated as

IC_{50} OA /IC_{50} DTX-1 (or DTX-2) × 100. This procedure identified a MAb OUB-OA-7, which was 100% cross-reactive with DTX-1 and 60% cross-reactive with DTX-2 in comparison with OA and with IC_{50}s of below 10 ng/ml for all three toxins. The cross-reactivity profile of this MAb correlated exactly with the TEFs of the OA group of toxins and was considered an excellent candidate for assay development on the optical biosensor platform to deliver a unique antibody-based screening assay. Preliminary studies in matrix using QUB-OA-7 determined that OA, DTX-1 and DTX-2 could all be detected at levels lower than the current EU regulatory limit and that the cross-reactivities of the antibody had not changed significantly (Table 4).

Table 3
Mid points on standard curves of the seven selected monoclonal antibodies.

Monoclonal antibody	IC_{50} OA
QUB-OA-1	8.5
QUB-OA-2	14.1
QUB-OA-3	40.1
QUB-OA-4	41.7
QUB-OA-5	31.8
QUB-OA-6	17.7
QUB-OA-7	4.8

Table 4
Cross-reactivity profiles of the monoclonal antibodies in buffer and following preliminary matrix extraction procedure.

Monoclonal antibody	IC_{50} (ng/ml)			Percentage cross-reactivity relative to OA	
	OA	DTX-1	DTX-2	DTX-1	DTX-2
Buffer QUB-OA-1 ($n = 6$)	8.5	8.7	8.5	100	100
QUB-OA-7 ($n = 4$)	4.8	4.9	8	100	60
Matrix QUB-OA-7 ($n = 2$)	80	89	113	90	71

During the screening of the present fusion a single lane of the CM5 sensor chip was used repeatedly, performing in excess of 600 analytical cycles without any decrease in performance. This data strongly suggests the surface chemistry employed in this study will be suitable for the assay development.

4. Conclusions

The current mammalian assays officially prescribed as the reference methods for the detection of the OA group of toxins have limited capability to detect these toxins at the present EU regulatory level of 160 μg OA equivalents/kg shellfish meat and are not capable of detecting OA group toxins below this level. Any replacement procedure for the MBA must be able to factor in the relative toxicities of all analogues that contribute to consumer health risk. Immuno-based formats allow for high sample throughput but the antibodies employed in the OA immunoassays described to date have limited data regarding cross-reactivity with all OA structurally related toxins. In this study a panel of MAbs was generated to OA with the aim of developing an SPR-based biosensor assay for detection of all OA toxins. An automated biosensor assay was developed and compared with the commonly used ELISA assay formats for screening of the hybridomas produced. Screening by biosensor was found to have considerably more advantages than ELISA: (a) no false positives results were detected, (b) it was the most efficient assay system in terms of toxin usage and time required for the assay; and (c) it provided immediate information on antibody binding characteristics.

Incorporation of characterisation studies into the screening assay identified two very sensitive MAbs antibodies, QUB-OA-1 and QUB-OA-7. QUB-OA-7 was subsequently found to have a cross-reactivity profile in buffer which correlated exactly with the intrinsic toxic potency of the OA group of toxins. The pilot matrix studies consolidated this MAb antibody as an excellent candidate for full biosensor assay development as this antibody has the potential to detect all OA toxins that contribute to major contamination outbreaks relative to their TEFs.

Please cite this article in press as: Stewart, L.D., et al., Development of a monoclonal antibody binding okadaic acid and dinophysistoxins-1, -2 in proportion to their toxicity equivalence factors, Toxicon (2009), doi:10.1016/j.toxicon.2009.05.015

Further assay development, matrix studies and inter-assay validation studies are required to determine the suitability of this antibody as a potential candidate for routine monitoring purposes. The biosensor-based assay could be used for preliminary screening of the toxicity of shellfish samples and if the sample is considered suspicious, complementary analytical techniques could then be used in parallel in order to provide a full toxin determination and quantification. An additional benefit of the biosensor procedure is that results obtained are semi-quantitative which can give better management information on whether the toxin levels found in a sample are low, close to, or above the maximum permitted level. Regulatory authorities and shellfish producers could use this additional data to make judgements relating to sampling levels and harvesting.

Acknowledgements

The authors would like to express their thanks to Ms Laura Plumpton, Dr Rodat Cunningham and Ms Joanne McGrath for their technical help during the study and Dr Philipp Hess for the supply of DTX-2. We also acknowledge financial support from the European Commission under the Sixth Framework Programme Priority 5 Food Quality.

Conflict of interest

The authors declare that there are no conflicts of interest.

References

Amzil, Z., Pouchus, Y.F., Le Boterff, J., Roussakis, C., Verbist, J.-F., Marcaillou-Lebaut, C., Masselin, P., 1992. Short-time cytotoxicity of mussel extracts: a new bioassay for okadaic acid detection. Toxicon 30, 1419–1425.

Aune, T., Yasumoto, T., Engeland, E., 1991. Light and scanning electron microscopic studies on effects of marine algal toxins toward freshly prepared hepatocytes. J. Toxicol. Environ. Health 34, 1–9.

Aune, T., Larsen, S., Aasen, J.A.B., Rehmann, N., Satake, M., Hess, P., 2007. Relative toxicity of dinophysistoxin-2 (DTX-2) compared with okadaic acid, based on acute intraperitoneal activity in mice. Toxicon 49 (1), 1–7.

Campas, M., Prieto-Simon, B., Marty, J., 2007. Biosensors to detect marine toxins: assessing seafood safety. Talanta 72, 884–895.

Campbell, K., Stewart, L.D., Doucette, G.J., Fodey, T.L., Haughey, S.A., Vilarino, N., Kawatsu, K., Elliott, C.T., 2007. Assessment of specific binding proteins suitable for the detection of paralytic shellfish poisons using optical biosensor technology. Anal. Chem. 79, 5906–5914.

Carmody, E.P., James, K.J., Kelly, S.S., 1996. Dinophysistoxin-2: the predominant diarrhoetic shellfish toxin in Ireland. Toxicon 34 (3), 351–359.

Connolly, L., Thompson, C.S., Haughey, S.A., Traynor, I.M., Tittlemeier, S., Elliott, C.T., 2007. The development of a multi-nitroimidazole residue analysis assay by optical biosensor via a proof of concept project to develop and assess a prototype test kit. Anal. Chim. Acta 598, 155–161.

EC, 2002. Commission Decision 2002/225/EC of 15 March 2002 laying down detailed rules for the implementation of Council Directive 91/492/EEC as regards the maximum levels and the methods of analysis of certain marine biotoxins in bivalve mollusc, echinoderms, tunicates and marine gastropods. Off. J. Eur. Commun. L75, 62–66.

Elliott, C.T., Baxter, G.A., Crooks, S.R.H., McCaughey, W.J., 1999. The development of a rapid immunobiosensor screening method for the detection of residues of sulphadiazine. Food Agric. Immunol 11, 19–27.

Ferguson, J.P., Baxter, G.A., McEvoy, J.D.G., Stead, S., Rawlings, E., Sharman, M., 2002. Detection of streptomycin and dihydrostreptomycin residues in milk, honey and meat samples using an optical biosensor. Analyst 127, 951–956.

Ferguson, J., Baxter, A., Young, P., Kennedy, G., Elliott, C., Weigel, S., Gatermann, R., Ashwin, H., Stead, S., Sharman, M., 2005. Detection of chloramphenical and chloramphenicol glucuronide residues in poultry muscle, honey, prawn and milk using a surface plasmon resonance biosensor and Qflex® kit chloramphenicol. Anal. Chim. Acta 529, 109–113.

Garcia, C., Truan, D., Lagos, M., Santelices, J.P., Diaz, J.C., Lagos, N., 2005. Metabolic transformation of dinophysistoxin-3 into dinophysistoxin-1 causes human intoxication by consumption of *O*-acyl-derivatives dinophysistoxins contaminated shellfish. J. Toxicol. Sci. 30, 287–296.

Gaudin, V., Fontaine, J., Maris, P., 2001. Screening of penicillin residues in milk by a surface plasmon resonance-based biosensor assay: comparison of chemical and enzymatic sample pre-treatment. Anal. Chim. Acta 436, 191–198.

Gaudin, V., Cadieu, N., Sanders, P., 2005. Results of a European proficiency test for the detection of streptomycin/dihydrostreptomycin, gentamicin and neomycin in milk by ELISA and biosensor methods. Anal. Chim. Acta 529, 273–283.

Gustavsson, E., Bjurling, P., Degelaen, J., Sternesjö, Å., 2002. Analysis of β-lactam antibiotics using a microbial receptor protein-based biosensor assay. Food Agric. Immunol 14, 121–131.

Haasnoot, W., Loomans, E., Cazemier, G., Dietrich, R., Verheijen, R., Bergwerff, A.A., Stephany, R.W., 2002. Direct versus competitive biosensor immunoassays for the detection of (dihydro)streptomycin residues in milk. Food Agric. Immunol 14, 15–27.

Hallegraeff, G.M., 1995. Harmful algal blooms: a global overview, in: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae, IOC Manuals and Guides No. 33. UNESCO, pp. 1–22.

Hess, P., Grune, B., Anderson, D.B., 2006. Three Rs approaches in marine biotoxin testing. ATLA 34, 193–224.

Honkanen, R.E., Mowdy, D.E., Dickey, R.W., 1996. Detection of DSP-toxins, okadaic acid, and dinophysistoxin-1 in shellfish by serine/threonine protein phosphatase assay. J. AOAC Int 79, 1336–1343.

Isobe, M., Sugiyama, Y., Ito, T., Ohtani, I.I., Toya, Y., Nishigohri, Y., Takai, A., 1995. New analysis method for protein phosphatase type 2a inhibitors using the firefly bioluminescence system. Biosci. Biotech. Biochem 12, 2235–2238.

James, K.J., Bishop, A.G., Carmody, E.P., Kelly, S.S., 2000. Detection methods for okadaic acid and analogues. In: Botana, L.M. (Ed.), Seafood and Freshwater Toxins: Pharmacology, Physiology and Detection. Marcel Dekker, New York, pp. 217–238.

Jorgensen, K., Scanlon, S., Jensen, L.B., 2005. Diarrhetic shellfish poisoning toxin esters in Danish blue mussels and surf clams. Food Add. Contam 22 (8), 743–751.

Kohler, G., Milstein, C., 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256 (5517), 495–497.

Kreuzer, M.P., O'Sullivan, C.K., Guilbert, G., 1999. Development of an ultra sensitive immunoassay for rapid measurement of okadaic acid and its isomers. Anal. Chem. 71, 4198–4202.

Llamas, N.M., Stewart, L., Fodey, T., Higgins, H.C., Velasco, M.L.R., Botana, L. M., Elliott, C.T., 2007. Development of a novel immunobiosensor method for the rapid detection of okadaic acid contamination in shellfish extracts. Anal. Bioanal. Chem. 389, 581–587.

Luu, H.A., Chen, D.Z., Magoon, J., Worms, J., Smith, J., Holmes, C.F., 1993. Quantification of diarrhetic shellfish toxins and identification of novel protein phosphatase inhibitors in marine phytoplankton and mussels. Toxicon 31, 75–83.

Mauriz, E., Calle, A., Abad, A., Montoya, A., Hildebrandt, A., Barceló, D., Lechuga, L.M., 2006. Determination of carbaryl in natural water samples by a surface plasmon resonance flow-through immunosensor. Biosens. Bioelectron 21, 2129–2136.

McNabb, P., 2008. Chemistry, metabolism, and chemical analysis of okadaic acid group toxins. In: Botana, L.M. (Ed.), Seafood and Freshwater Toxins: Pharmacology, Physiology and Detection, second ed. Marcel Dekker, New York, pp. 209–228.

Mello, L.D., Kubota, L.T., 2002. Review of the use of biosensors as analytical tools in the food and drink industries. Food Chem. 77, 237–256.

Quilliam, M.A., Wright, J.L.C., 1995. Methods for diarrhetic shellfish poisons, in: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae. IOC Manuals and Guide No. 33. UNESCO, pp. 95–111.

Samsonova, J.V., Baxter, A., Crooks, S.R.H., Small, A.E., Elliott, C.T., 2002. Determination of ivermectin in bovine liver by optical immunobiosensor. Biosens. Bioelectron 17, 523–529.

Please cite this article in press as: Stewart, L.D., et al., Development of a monoclonal antibody binding okadaic acid and dinophysistoxins-1, -2 in proportion to their toxicity equivalence factors, Toxicon (2009), doi:10.1016/j.toxicon.2009.05.015

Simon, J.F., Vernoux, J.P., 1994. Highly sensitive assay of okadaic acid using protein phosphatase and paranitrophenyl phosphate. Nat. Toxins 2, 293–301.

Torgersen, T., Aasen, J., Aune, T., 2005. Diarrhetic shellfish poisoning by okadaic acid esters from Brown crabs (*Cancer pagurus*) in Norway. Toxicon 46, 572–578.

Traynor, I.M., Plumpton, L., Fodey, T.L., Higgins, C., Elliott, C.T., 2006. Immunobiosensor detection of domoic acid as a screening test in bivalve molluscs: Comparison with LC based analysis. J. AOAC Int 89, 868–873.

Tubaro, A., Florio, C., Luxich, E., Vertua, R., Della Loggia, R., Yasumoto, T., 1996a. Suitability of the MTT-based cytotoxic assay to detect okadaic acid contamination in mussels. Toxicon 34, 965–974.

Tubaro, A., Florio, C., Luxich, E., Sosa, S., Della Loggia, R., Yasumoto, T., 1996b. A protein phosphatase 2a inhibition assay for a fast and sensitive assessment of okadaic acid contamination in mussels. Toxicon 34, 743–752.

Vale, P., Sampayo, M., 2002. First confirmation of human diarrhoeic poisonings by okadaic acid esters after ingestion of razor clams (*Solen marginatus*) and green crabs (*Carcinus maenas*) in Aveiro lagoon, Portugal and detection of okadaic acid esters in phytoplankton. Toxicon 40, 989–996.

Vieytes, M.R., Fontal, O.I., Leira, F., Baptista de Sousa, J.M.V., Botana, L.M., 1997. A fluorescent microplate assay for diarrheic shellfish toxins. Anal. Biochem 248, 258–264.

Villar-Gonzalez, A., Rodriguez-Velasco, M.L., Ben-Gigirey, B., Botana, L.M., 2007. Lipophilic toxin profile in Galacia (Spain): 2005 toxic episode. Toxicon 49, 1129–1134.

Yasumoto, T., Oshima, Y., Yamaguchi, M., 1978. Occurrence of a new type of shellfish poisoning in the. Tohuku District. Bull. Jpn. Soc. Fish 44, 1249–1255.

Zilka, N., Vechterova, L., Kontsekove, E., Novak, M., 2003. A rapid immunohistochemical primary screening assay for hybridomas. J. Immunol. Methods 272, 49–53.

Please cite this article in press as: Stewart, L.D., et al., Development of a monoclonal antibody binding okadaic acid and dinophysistoxins-1, -2 in proportion to their toxicity equivalence factors, Toxicon (2009), doi:10.1016/j.toxicon.2009.05.015