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Generic microcystin immunoassay based on monoclonal antibodies against Adda

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A monoclonal antibody (clone AD4G2) was generated against a common part of microcystins and nodularins, the unusual amino acid Adda [(2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4*E*,6*E*-dienoic acid]. A direct competitive ELISA based on this antibody was developed and the cross-reactivity pattern was measured. Different toxins showed a very similar response. The assay provides therefore a sum parameter of microcystins, nodularins and peptide fragments containing Adda. The IC_{50} for microcystin-LR was 0.33 μg L^{-1} which leads to a detection limit of 0.07 μg L^{-1}. This is well below the concentration of 1 μg L^{-1} proposed by the World Health Organisation (WHO) as the limit for drinking water. Microcystin-LR spiked water samples in the concentration range between 0.1 and 1 μg L^{-1} were measured and a mean recovery of 113 ± 23% was found. The antibody is well suited for the determination of microcystins in drinking as well as surface water.

Introduction

Microcystins (MC; Fig. 1) are highly toxic cyclic heptapeptides produced by some species of the cyanobacteria genera *Microcystis*, *Oscillatoria*, *Planktothrix*, *Anabaena* and *Nostoc*.[1–3] As cyanobacteria (blue-green algae) can occur in drinking water reservoirs and recreational lakes, microcystins represent an acute and chronic risk for human health. The determination of microcystins is demanding due to the high variability of the microcystin structure (currently up to 90 variants are known[4,5]) and the required sensitivity for analysis. For toxicological reasons all microcystins must be detected, but, however, only few of them are commercially available. An analytical system is therefore required to detect all microcystins equally and give a sum concentration of them.

Immunoassays using antibodies with broad specificity are suitable analytical methods for this purpose. Several polyclonal and some monoclonal antibodies have been generated against microcystins.[6–13] Most of the research groups used microcystin-LR as a hapten for immunisation, one used microcystin-LA.[14] In this work, another immunisation strategy was applied for generation of monoclonal antibodies specific to microcystins. Solely a characteristic part of the peptides, Adda [(2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4*E*,6*E*-dienoic acid], coupled to a carrier protein was used for immunisation (Fig. 2). The rare amino acid, Adda, is common to all microcystins and nodularins. In contrast to proteinogenic amino acids, Adda has a long hydrophobic side chain. The size and shape of the molecule should be large and distinct enough to generate antibodies with high affinity.

A similar immunisation strategy was attempted by Baier *et al.*[11] They used an Adda precursor [(6*S*,7*S*,2*E*,4*E*)-7,*N*-dimethoxy- 4,6,*N*-trimethyl-8-phenyl-2,4-octanoic acid amide] without coupling to a carrier protein and found a strong immune response to the Adda precursor but not to microcystins. Attempts to generate class specific antibodies by immunisation with a generic substructure of the molecule were published for other analytes, such as sulfonamides[15] or sulfonylurea herbicides,[16] as well. Unfortunately, the success of this approach was limited, as some compounds were discriminated against. This might be due to the small size of the immunising haptens and the spacer introduced between hapten and carrier protein.

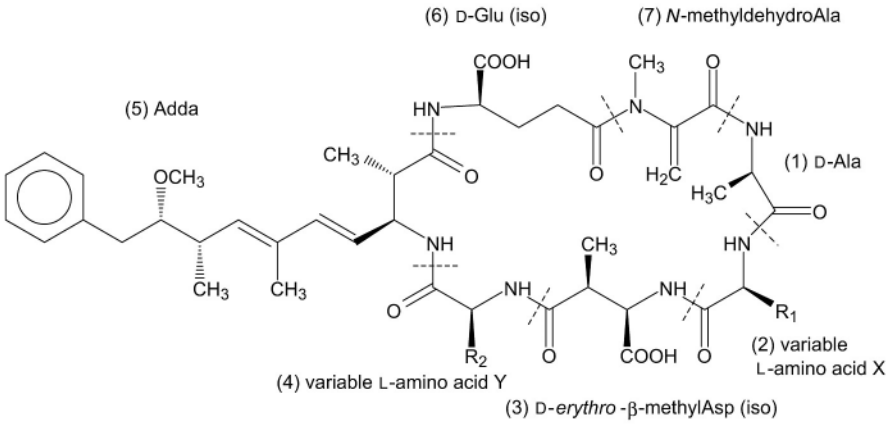

Fig. 1 General structure of microcystins [cyclo(-D-Ala-L-X-D-*erythro*-β-methyl-iso-Asp-L-Y-Adda-D-iso-Glu-*N*-methyldehydroAla)]. Other variations in the structure are the methylation of the carboxylic acids (3) and (6), the demethylation of the amide (7) and the side chain of (3), methylation at the side chain of (7) and changes in the all-*trans* conformation of the conjugated double bonds of Adda.

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Materials and methods

Microcystins and their derivatives

The general structure of microcystins (Fig. 1) is cyclo(D-Ala-L-X-D-*erythro*-β-methyl-iso-Asp-L-Y-Adda-D-iso-Glu-*N*-methyldehydroAla), where X and Y are the one-letter codes of the variable amino acids (microcystin-XY). [D-Asp^3,Dhb^7]MC-RR, [D-Asp^3]MC-HtyrR, [D-Asp^3]MC-LR, [D-Asp^3]MC-RR, (Htyr; homotyrosine) MC-LY and MC-WR were purchased from Anagnostec (Luckenwalde, Germany). According to Anagnostec, MC-LY was isolated from an Asian strain, MC-WR from an Asian field sample, [D-Asp^3,Dhb^7]MC-RR (Dhb; dehydrobutyrine) from a German cyanobacterial bloom, [D-Asp^3]MC-RR from *Planktothrix agardhii*, [D-Asp^3]MC-LR from *Microcystis aeruginosa* and [D-Asp^3]MC-HtyrR from *Planktothrix rubescens*. All microcystins purchased from Anagnostec had been tested by matrix-assisted laser desorption-ionisation mass spectrometry. MC-LR (purity ≥98%), MC-RR (purity ≥97%), MC-LF (purity ≥95%) and MC-LW (purity ≥95%) isolated from *Microcystis aeruginosa* were supplied by Alexis (Läufelfingen, Switzerland, purity tested by HPLC, 238 nm). MC-RR, MC-YR, MC-LA isolated from *Microcystis aeruginosa* and nodularin-R isolated from *Nodularia spumigena* (all ≥95% by HPLC) were supplied by Calbiochem (La Jolla, CA, USA). Adda was kindly provided by Dr. D. Cundy and Dr. T. McCarthy (CSIRO Molecular Science, Clayton South, Victoria, Australia). The synthesis of Adda and Adda-containing dipeptides is described by Cundy *et al*.[17,18] The synthesis of *N*-Ac–Adda is described by Zeck *et al*.[13]

Reagents and chemicals

Keyhole limpet hemocyanin (KLH) subunits (M_r 370 kDa) were obtained as a stock solution (Vacmun) by Biosyn (Fellbach, Germany). Horseradish peroxidase (HRP; ELISA grade) was obtained from Roche Diagnostics (Mannheim, Germany). Bovine serum albumin (fraction V, purity 99%) was obtained from Merck (Darmstadt, Germany), goat anti-mouse IgG (F_c fragment) from ICN Pharmaceuticals (Eschwege, Germany) and horse–anti-mouse–horseradish peroxidase conjugate from Vector (Burlingame, CA, USA). All other chemicals were obtained from Sigma-Aldrich-Fluka (Steinheim, Germany) or Merck (Darmstadt, Germany). Buffers and standards were prepared in purified, UV-treated water (Milli-Q plus 185, Millipore). The monoclonal antibody M8H5 was kindly provided by Dr. Nagata and Prof. Ueno (Tokyo, Japan).

Apparatus

Flat bottom polystyrene 96-well immunoplates with high binding capacity were purchased from Greiner (Nürtingen, Germany). A Columbus washer for microtiter plates, an Easyshaker EAS 2/4 and a Reader 340 ATTC for microtiter plates controlled by a personal computer containing the standard software package EasySoftware from SLT (Gröding/Salzburg, Austria) were used. All data processing was done with Origin 6.0 (Microcal Software Inc., Northhampton, USA). HPLC measurement was performed with a L-6200A Intelligent Pump and an UV/VIS detector L-4250 from Merck using ChromStar software from SCPA (Stuhr, Germany) for data collection. Mass spectra were obtained with an electrospray time-of-flight mass spectrometer (ESI-TOF MS), LCT, Micromass (Manchester, UK). Direct injection (18 μL min^{-1}) was achieved with a syringe pump, model 11 from Harvard Apparatus (Holliston, MA, USA). The parameters used for ESI-TOF were: Polarity: ES+; capillary voltage: 3400 V; sample cone voltage: 30 V; RF lens: 350 V; extraction cone voltage: 5 V; source temperature: 100 °C; desolvation temperature: 350 °C; nebulizer gas flow: 99 L h^{-1}; desolvation gas flow: 436 L h^{-1}.

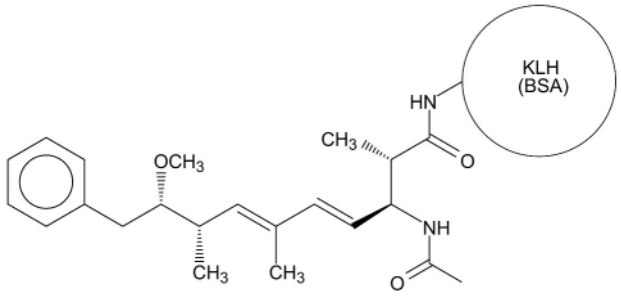

Fig. 2 Immunogen for generation of monoclonal antibodies with broad specificity to microcystins.

Direct competitive ELISA protocol for the monoclonal antibody AD4G2

The 96-well microtitration plates were coated overnight at room temperature (RT) with 250 μL anti-mouse IgG (F_c-specific) serum diluted 1:3000 in coating buffer (40 mM carbonate, pH 9.6). After washing with PBS (pH 7.6, plus 0.05% Tween 20), the wells were incubated with the diluted cell culture supernatant of monoclonal antibody AD4G2 (200 μL, about 12 μg L^{-1} in PBS) for 3 h at RT. After a further washing step, PBS buffer (20 μL per well) and subsequently microcystin standards in the concentration range from 0.001 to 1000 μg L^{-1} or samples (200 μL per well) were added to the plate. The microcystin stock solutions (1 g L^{-1}) were prepared in Milli-Q water and their concentration calibrated at a UV absorbance of 238 nm using the molar absorption coefficient of MC-LR ε = 39 800 (L·mol^{-1}·cm^{-1}).[19] After a preincubation of the plate for 1 h, peroxidase tracer (*N*-Ac–Adda–HRP conjugate, 50 μL per well, 0.2 mg L^{-1} in PBS) was added for an additional 15 min. The microtitration plate was subsequently washed and substrate solution (tetramethylbenzidine, hydrogen peroxide, citrate buffer pH 3.8, 200 μL per well) was added. After a suitable development time (up to 30 min) the reaction was stopped with dilute sulfuric acid (5%, 100 μL per well). Subsequently absorbance was measured at 450 nm (reference wavelength 620 nm) with a microplate reader.

For measurement of water samples, a modified protocol was used as described by Zeck *et al*.[13] Briefly, TRIS buffer (pH 7.4, 0.1 mol L^{-1}) containing 0.1% BSA and 0.1% EDTA replaced PBS buffer for tracer sample dilution. For sample dilution a ten-fold concentrated buffer was used. Sample dilution was performed in the wells of the plate by adding first the buffer (20 μL per well) and then the samples (200 μL per well).

Evaluation of standard curves and measurement of cross-reactivity

Each calibration point of standard curves and each sample value was determined by calculation of the median of the data (n = 3 or 4). Standard curves of different microcystin variants were obtained by fitting calculated medians to a four-parameter function.[20] The detection limit for MC-LR was calculated according to the 3s-definition using a 17%-trimmed mean[21] of the blank (n = 12). Cross-reactivities (CR) were calculated using the midpoints of standard curves (CR = [$IC_{50,MC\text{-}LR}$/$IC_{50,MC\text{-}XY}$] × 100). For molar cross-reactivities, all concentrations were calculated in mol L^{-1}.

Indirect, competitive ELISA for monitoring the immune response of the *N*-Ac–Adda–KLH immunised mice

The wells of a microtitration plate were incubated for 6 h with *N*-Ac–Adda–BSA (200 μL per well, 70 μg L^{-1} in coating buffer). After a washing step, the wells were blocked with 1% (m/v) casein (250 μL per well, in PBS) for 30 min. After a further washing step, Adda or microcystin standards (100 μL per well) and subsequently the antisera (100 μL per well, 1:5000 diluted in PBS) were added to the wells and incubated for one hour. The secondary antibody (200 μL per well, anti-mouse–HRP conjugate, diluted 1 : 50 000 in PBS) was added to the wells after a further washing step. Signal development was performed as described above.

Screening of cell culture supernatants

The culture fluids of the growing hybridoma cells and of the recloned cells were tested by a direct, non-competitive ELISA. Precoating with anti-mouse antibody was done as described for the direct competitive ELISA procedure. After a washing step, the plates were incubated for 2 h with cell culture supernatant (200 μL per well, diluted 1 : 40 in PBS, 2 h, RT). As a positive control, the polyclonal serum of the positive mouse was included (1:670) on the plate. Then, the plates were washed and *N*-Ac–Adda enzyme tracer (200 μL per well, diluted 1 : 10 000 in PBS) was added. After 1 h, the plates were washed and 200 μL per well of freshly prepared enzyme substrate solution was added. Signal development was performed as described above.

Synthesis of *N*-Ac–Adda–protein conjugates (proteins: KLH, BSA, and HRP)

N-Ac–Adda (4.2 mg; 11.3 μmol) was dissolved in 400 μL of dry glyme (1,2-dimethoxyethane). *N*-hydroxysuccinimide (1.5 mg; 13 μmol) and di(*N*-succinimidyl)-carbonate (5.7 mg; 22 μmol) were added and stirred for 1 h at RT. Then, dicyclohexylcarbodiimide (4 mg; 20.8 μmol) was added to the mixture and stirred overnight at RT. The reaction mixture was centrifuged and the supernatant was concentrated to 200 μL under a stream of nitrogen, mixed with 200 μL water and purified immediately by semipreparative HPLC on Supelcosil C18 (5 μm, 25 cm × 1 cm). Gradient (A: water; B: acetonitrile; flow: 6 mL min^{-1}): 0 min to 1 min 70% A; 30 min 20% A; 35 min 10% A; 45 min 70% A, wavelength: 238 nm, retention time of *N*-Ac–Adda: 14 min.; *N*-Ac–Adda–NHS: 19 min. (NHS; *N*-hydroxysuccinimide). The reaction product peak was collected and immediately frozen at –23 °C. The yield of purified activated *N*-Ac–Adda ester was 30% according to chromatography (UV at 238 nm). The liquid acetonitrile phase containing the *N*-Ac–Adda–NHS ester was decanted from the frozen water phase. The water phase was extracted with acetonitrile a further two times. The acetonitrile phases were combined and dried immediately with Na_2SO_4.

A stock solution of KLH subunits (245 μL of a 20.4 g L^{-1} solution; 0.46 μmol amino groups) was mixed with borate buffer (500 μL; 0.1 M; pH 9.2) and *N*-Ac–Adda–NHS (approx. 0.57 μg or 1.2 μmol respectively in 230 μL of acetonitrile) was added in aliquots of 10 μL to the KLH solution. After one hour of stirring at room temperature, the solution became cloudy and the protein conjugate precipitated. The precipitate was centrifuged and washed once with 500 μL of methanol (+0.1% acetic acid) and once with pure methanol. The reaction supernatant was found to contain trace amounts of protein which were used to prove the coupling success. An indirect non-competitive ELISA with the soluble *N*-Ac–Adda–KLH as coating conjugate and M8H5 as primary antibody was performed and showed a high signal in comparison to the blank (KLH as coating antigen).

The coupling of the *N*-Ac–Adda–NHS (activated ester) to BSA and HRP respectively was performed similar to the coupling to KLH. The BSA and HRP conjugates remained soluble and were purified by gel chromatography (Sephadex PD-10, Pharmacia, Uppsala, Sweden). The number of *N*-Ac–Adda molecules coupled to BSA was determined by ESI-TOF MS to be 10. The coupling density was therefore 28% assuming a total number of 36 accessible primary amino groups in BSA.[22]

Production of monoclonal antibodies

Immunisation of mice. Generation of hybridoma was performed by Connex (Martinsried, Germany). Three mice (Balb/cxC57, 6–12 weeks old) were immunised intraperitoneally (i.p.) with 7.5 μg *N*-Ac–Adda–KLH (crushed fine powder, suspended in PBS to a final concentration of 5.5 g L^{-1}) solution emulsified with Freund′s Complete Adjuvant and boosted with Freund′s Incomplete Adjuvant (twice) in intervals of five weeks. Blood was collected eight to ten days after the immunisations and tested by an indirect ELISA for antibodies against *N*-Ac–Adda coupled to BSA. After the third immunisation, a carrier switch was performed and further immunisations were done with 7.5 μg of soluble *N*-Ac–Adda–BSA conjugate keeping the same intervals between booster injections and blood collection. The sera were tested by a direct ELISA using *N*-Ac–Adda–HRP conjugate as tracer and different competitors as analytes.

Fusion and cloning. To produce hybridoma cells, two positive mice were primed intraperitoneally three times on consecutive days with 10 μg (mouse 217) and 15 μg (mouse 218) soluble immunogen without adjuvant. On day five, the spleen was isolated, spleen cells were fused with myeloma cells (P3 × 63-Ag8.653) and suspended in HAT (hypoxanthine aminopterin thymidine) selection medium. Then, they were plated on 96-well tissue cell culture plates at 50 000 cells per well. The culture fluids of the wells containing growing hybridoma cells were screened by direct ELISA for antibodies recognising the *N*-Ac–Adda–HRP enzyme tracer. Positive cultures were immediately cloned by limiting dilution (average number of 10 cells per well for the first recloning and 1 cell per well for the second and third recloning) until stable. After sowing an average number of only one cell per well, the direct ELISA signal of the cell culture supernatant was very low after one week. Multiple subsequent measurements were required to confirm the positive results. If an average number of 10 cells per well were sown, antibodies could be easily detected in the cell culture fluid after only one week. The immunoglobulin subclass of monoclonal antibodies was determined by the Mouse Monoclonal Antibody Isotyping Kit (IsoStrip) of Roche Diagnostics (Mannheim, Germany).

Results and discussion

Immunogen design and synthesis for anti-microcystin antibodies of broad specificity

The purpose of the work was the generation of monoclonal antibodies with broad specificity against microcystins. Therefore, a derivative of the amino acid Adda was chosen as hapten. Adda is part of the common substructure of all microcystins and nodularins. To prevent strong recognition of the positively charged amino group by the antibody binding site, this group was blocked by acetylation. Together with the derivatization of

the carboxylic group by coupling to the carrier protein, the structure of the hapten is very similar to that of Adda as part of microcystins (compare Fig. 1 and Fig. 2).

No spacer was used between Adda and the carrier protein to limit steric accessibility of Adda and to prevent its binding in a deep binding pocket. Binding of Adda in a deep crevice could eventually lead to non-binding of microcystins as the bulky cyclic peptide ring would not fit into the binding pocket.

The coupling of the hapten to the proteins KLH, BSA and HRP was achieved through an amide bond between the carboxylic group of Adda and primary amino groups of the protein using the activated ester method. Purification of the activated *N*-Ac–Adda ester by semipreparative HPLC was performed to prevent the reaction of a surplus of carbodiimide with proteins. The reaction of carbodiimides with functional groups of proteins yields stable *N*-acylurea and guanidine structures, which can be recognised by the immune system.[23] This would lead to non-specific signal development in the indirect and direct ELISA format when the coating antigen and the enzyme tracer were synthesised by the same procedure. The structure of the immunogen is shown in Fig. 2. The *N*-Ac–Adda–KLH was largely insoluble whereas the *N*-Ac–Adda–BSA immunogen was soluble.

During immunisation, antisera were examined for binding of the *N*-Ac–Adda and the microcystin-LR enzyme tracer as well as for inhibition by *N*-Ac–Adda, Adda, MC-LR and -LA. Microcystin-LR was chosen as a representative for those microcystins containing a positive charge close to Adda and inversely, microcystin-LA as a representative for microcystins without a charge in the neighbourhood of Adda.

Monitoring the immune response against the *N*-Ac–Adda–KLH and –BSA conjugates

To obtain high affinity antibodies, a rather low dose of conjugate of 7.5 μg *N*-Ac–Adda–KLH per boost was chosen. A low antigen dose is known to be favourable for the derivation of high affinity antibodies.[24] Similar to the immunisation schedule applied for generation of monoclonal microcystin antibodies,[13] the first booster injections were done with insoluble immunogen which presumably has a poorer availability for the immune system but is more stable against degradation than the soluble immunogen. Immunising the insoluble immunogen gave only low antibody titers. After the first booster injection, all mice were positive in the indirect ELISA, but not in the direct ELISA. This can be explained by the low affinity of the antibodies originating from the first antisera. After the second booster injection, an affinity maturation was observed leading to a decrease of the IC_{50} from 40 to 11 μg L^{-1} (MC-LR, mouse 217). This affinity allowed the performance of a direct ELISA, which showed an IC_{50} of 1.6 μg L^{-1}. After the third immunisation, no additional affinity maturation could be observed and the antibody titer remained very low. At this stage, a carrier switch (B in Fig. 3) to a soluble immunogen was performed to obtain a higher concentration of specific antibodies.

Fig. 3 shows a comparison between the immunisation of two mice against MC-LR–cOVA (cationized ovalbumin)[13] and *N*-Ac–Adda–BSA, respectively. Both were started with low doses of insoluble immunogen and gave low antisera titer but high affinity antibodies in relation to their hapten structure. For the generation of the MC-LR antibodies, a switch from insoluble to soluble conjugate was performed using the same carrier protein (cOVA). In this case, a jump in the specific antibody concentration appeared on average one booster injection earlier than in the case of the *N*-Ac–Adda immunisation, where simultaneously a carrier switch was performed. A reason for the delayed immune response of the latter could be the stimulation of different helper T-cells due to different processing products presented by the specific B-cells. Nevertheless, as the midpoints did not change after the carrier switch, some evidence exists, that still the same B-cells were activated by new helper T-cells. Fig. 3 demonstrates that a carrier switch results in a delay of a strong immune response to haptens. Another point of interest was the investigation of the cross-reactivity pattern of the induced antibodies in different mice during immunisation. The midpoints obtained for *N*-Ac–Adda, MC-LR and MC-LA are listed in Table 1.

As Table 1 shows, the polyclonal antiserum of mouse 218 already showed a broad reactivity towards microcystins. The antiserum of mouse 217 showed a similar recognition pattern whereas the third mouse (219) had no significant immune response. These results support the assumption that the immunisation of a certain hapten structure induces the generation of antibodies with similar cross-reactivity pattern.

Isolation of hybridoma cell lines producing anti-Adda antibodies

To produce hybridoma cells, mouse 217 was boosted three times on consecutive days with 10 μg immunogen and killed on the 5th day. The spleen of this mouse was relatively small and presumably not swollen. This can be due to the low immunogen dose used for preparation of the fusion. Consequently, only 87 growing hybridoma cells were obtained and none of them was positive. Mouse 218 was boosted with 15 μg immunogen on three consecutive days and spleen was taken on the 5th day. This fusion yielded nearly 800 growing hybridoma cells. Only one cell colony was found to produce antibodies reacting with the *N*-Ac–Adda–HRP enzyme tracer (signal/noise 800). This cell colony was recloned three times. After the third recloning, one of the subclones was assumed to be genetically stable as 97% of the growing daughter cell colonies were positive. This

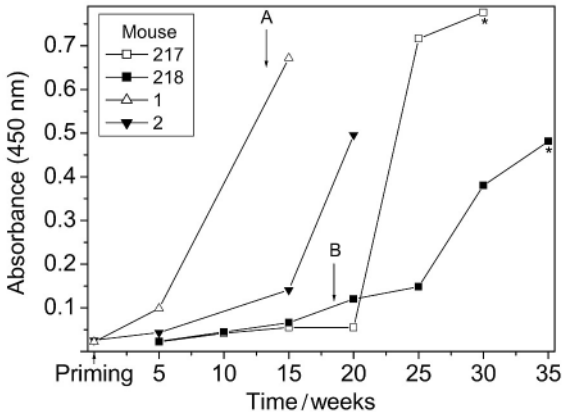

Fig. 3 Monitoring the antisera titer of mice 217, 218, 1 and 2. Mice 1 and 2 were immunised with MC-LR–cOVA conjugate[13] and a carrier switch from insoluble and soluble MC-LR–cOVA conjugate was performed at A. Mice 217 and 218 were first immunised with insoluble *N*-Ac–Adda–KLH and after B with soluble *N*-Ac–Adda–BSA. (*: Antiserum titer obtained from heart puncture two days after the last booster injection, just before fusion).

Table 1 Midpoints (IC_{50}) of *N*-Ac–Adda and of microcystins using the sera of the two mice (217 and 218, week 30) in the direct competitive ELISA (dilution of sera 1:500 in PBS)

Analyte	Midpoint (IC_{50})/ μg L^{-1} mouse 217	Midpoint (IC_{50})/ μg L^{-1} mouse 218
N-Ac–Adda	6.7 ± 0.4	1.4 ± 0.1
Microcystin-LR	4.1 ± 0.2	0.9 ± 0.1
Microcystin-LA	7.2 ± 1.8	1.1 ± 0.4

cell line was cultured to get larger amounts of antibody AD4G2 (IgG_1 with kappa light chain).

Standard curve (direct ELISA)

The new monoclonal antibody AD4G2 was generated against Adda with the purpose to measure microcystins. Therefore, as reference analyte, microcystin-LR was used instead of Adda. This has the additional advantage that comparisons with other antibodies are easier. Furthermore, microcystin-LR is one of the most common microcystins and the provisional guideline of the WHO refers to it. The standard curve for measurement of microcystin-LR with the monoclonal antibody AD4G2 is shown in Fig. 4.

The affinity of the antibody AD4G2 to microcystin-LR as shown by the midpoint of the calibration curve, is among the highest reported for microcystin antibodies. The detection limit of 0.07 μg L^{-1} makes this antibody suitable for measurement of microcystin-LR in accordance with the provisional guideline value of 1 μg L^{-1} microcystin-LR proposed by the WHO. As the antibody has a high affinity to microcystin-LR, it binds not only the *N*-Ac–Adda-, but also the microcystin-LR–enzyme tracer. Very similar standard curves were obtained with the different tracers.

Cross-reactivities

Fifteen cross-reactants were tested, inclusively Adda, two Adda derivatives and nodularin-R (Table 2). The molar cross-reactivities of the microcystins range between 50 and 120%, showing a very broad recognition pattern for microcystins. The midpoints of the standard curves of the cross-reactants are all below 1 μg L^{-1}, allowing a sensitive detection of all tested microcystins. For determination of the cross-reactivities, a microcystin-LR standard curve was measured in parallel and under the same conditions as a standard curve of the cross-reactant. Due to small changes of the midpoint for microcystin-LR, the cross-reactivities are not strictly correlated to the midpoints shown in Table 2. The inter-assay averaged midpoint (IC_{50}) for microcystin-LR was 0.39 ± 0.07 μg L^{-1} (n = 8; 1s).

Remarkable is the relatively low cross-reactivity of Adda and *N*-Ac–Adda as the latter was the hapten for immunisation. The only difference between this analyte and the immunogen is the presence of the negatively charged carboxylic acid. Indeed, the cross-reactivity of *N*-Ac–Adda–methylamide, of which the carboxylic group was converted into a methylamide, is 99%.

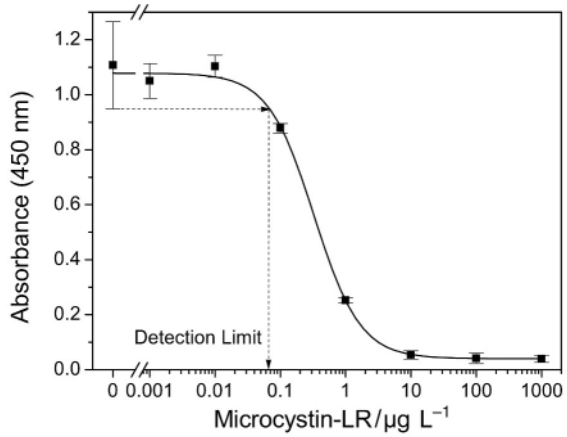

Fig. 4 Standard curve for measurement of microcystin-LR with the monoclonal antibody AD4G2 in the direct, competitive ELISA. Parameters: upper asymptote, 1.08; lower asymptote, 0.04; slope, 1.23; midpoint (IC_{50}), 0.33 μg L^{-1}; detection limit, 0.07 μg L^{-1}.

This supports the strong charge effects on the antigen–antibody binding.[20] Nevertheless, the midpoints of Adda and *N*-Ac–Adda are below 1 μg L^{-1} and even may allow a measurement of microcystin fragments.

Sample analysis

To demonstrate the application of the established ELISA to the analysis of water samples, water from different origins was spiked with MC-LR at three concentrations. Concentrations were selected to cover most of the measuring range. For measurement of real samples, a modified ELISA protocol was used, where TRIS buffer containing sodium chloride, EDTA and BSA replaced PBS buffer for sample conditioning and for tracer dilution.[25] BSA reduces the negative effects of humic acids to a minimum as they bind to the protein.[26] TRIS buffer and EDTA avoid the precipitation of the poorly soluble calcium phosphate in the case of hard-water samples. These changes have no effects on the calibration curve. In general it can be assumed that the stability of the antibody against interferences like organic solvents, oxidizing agents or surfactants is in the direct ELISA format similar to the stability data obtained for the antibodies MC10E7[13] and M8H5.[27] Both developed direct ELISAs with antibody MC10E7 and M8H5 revealed that the stability of the enzyme tracer[13] limits the susceptibility of the direct ELISA against interferences and not the stability of the antibody. Results of the water samples are listed in Table 3.

As the shape of the calibration curve is sigmoidal, the error is asymmetrical in relation to the average concentration. Some overestimation of low microcystin concentrations seems to occur. Nevertheless, Table 3 demonstrates that the direct ELISA is suitable for measurement of water samples with microcystin concentrations between 0.1 and 1 μg L^{-1}.

Conclusion

In this work, the generation of monoclonal antibodies with broad specificity to microcystins and nodularins is described. A novel immunisation strategy was successfully applied by immunisation of a generic substructure of the analyte molecule. As the substructure is common to the compound class to be measured, class specificity can be achieved. However, similar approaches have been applied with only limited success. This indicates that some additional conditions must be fulfilled. One condition might be the coupling of the hapten to the carrier

Table 2 Midpoints, detection limits and cross-reactivities of related analytes

Analyte	Midpoint/ μg L^{-1}	Detection limit/ μg L^{-1}	Molar Cross Reactivity (%)
Nodularin-R	0.16 ± 0.03	0.025	163 ± 0.41
Microcystin-YR	0.33 ± 0.02	0.043	120 ± 19
[D-Asp3]Microcystin-RR	0.23 ± 0.05	0.031	109 ± 31
Microcystin-LY	0.43 ± 0.05	0.057	103 ± 15
Microcystin-LR	0.33 ± 0.03	0.068	100
N-Ac–Adda–methylamide	0.14 ± 0.05	0.018	99 ± 54
[D-Asp3]Microcystin-LR	0.45 ± 0.03	0.095	97 ± 10
Microcystin-LW	0.43 ± 0.08	0.085	84 ± 32
Microcystin-WR	0.35 ± 0.07	0.043	76 ± 10
Microcystin-RR	0.46 ± 0.06	0.071	70 ± 16
Microcystin-LF	0.70 ± 0.08	0.135	69 ± 19
Microcystin-LA	0.42 ± 0.03	0.056	66 ± 24
[D-Asp3]Microcystin-HtyrR	0.39 ± 0.02	0.056	62 ± 8
[D-Asp3]Dhb7[Microcystin-RR	0.90 ± 0.07	0.212	51 ± 5
Adda	0.43 ± 0.10	0.093	27 ± 12
N-Ac–Adda	0.77 ± 0.03	0.143	25 ± 4

Table 3 Analysis of water samples

No.	Origin	MC-LR added to the sample/μg L^{-1}	MC-LR determined by ELISA[a] (range)/μg L^{-1}	Recovery of MC- LR (%)
1	Tap-water Munich/Germany	0.1	0.155 (0.126–0.185)	155
		0.5	0.527 (0.463–0.604)	105
		1	0.973 (0.859–1.123)	97
2	Wahnbachtalsperre near Bonn/Germany (drinking water reservoir)	0.1	0.141 (0.069–0.212)	141
		0.5	0.524 (0.510–0.538)	104
		1	0.901 (0.829–0.985)	90
3	Pucher See near Munich/Germany (lake)	0.1	0.129 (0.083–0.174)	129
		0.5	0.504 (0.483–0.526)	101
		1	0.945 (0.861–1.047)	95

[a] Based on the standard deviation (3*s*) of the 20%-trimmed values. Mean recovery 113 ± 23%.

protein without introduction of a spacer. This leads to antibodies without a deep binding pocket, which could discriminate larger molecules than the hapten.

Screening of the cell culture supernatants with the direct ELISA was very effective for recognition of positive clones. The multivalent binding between coating antigen and antibody in the indirect ELISA allows the detection of low affinity antibodies by taking advantage of the chelate effect. In the direct ELISA format, only antibodies with high affinity (IgG) can be detected due to the monovalent binding between HRP–enzyme tracer and antibody.

Using the new monoclonal antibody AD4G2, a direct competitive ELISA was established and the cross-reactivity pattern of the antibody was determined. The antibody was found to be suitable for measurement of microcystins in water samples in accordance with the demands of the provisional guideline value of 1 μg L^{-1} proposed by the WHO. In contrast to other microcystin antibodies with broad specificity,[27] this antibody recognises Adda and Adda derivatives with good sensitivity. Applications for the monitoring of the degradation of microcystins are therefore possible. In addition, the antibody might be used as a part of an immunosensor array.

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