R&D Project 348

Development of a field test kit for blue-green algal toxins

Biocode Ltd

December 1994

R&D Draft Final Report 348/6/A
(R&D Note)

• LSA
• People

DEVELOPMENT OF A FIELD TEST KIT FOR BLUE-GREEN ALGAL TOXINS

D Baron

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R&D Draft Final Report 348/6/A

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Dissemination List

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M Pearson - Anglian Region

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QUALITY REVIEW INVITATION

PM3/op

Project Reference: NRD 348 Function: Water Quality Region/H.O. Dept Anglian	Prepared by: G Brighty Date: 24 November 1994

Project Title	Stage *(if applicable)*
Development of field test kit for blue-green algal toxins	Draft Final Report

You are invited to attend a Quality Review of the following Product(s):

Product Code(s)	Product Name(s)
348/6/A	Draft Final Report - Development of a field test kit for detection of blue-green algal toxins

Venue (if postal enter "POSTAL")	Postal	Date Duration	19/12/ 94 **closes: 10/1/95**
Chairman	Paul Williams	Project Manager	M Pearson
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Product Description(s) attached

Draft Final Report summarising the work undertaken October 1992 to July 1994 on R&D project 348. Fully detailed Interim Reports (5 no) already presented and are bound as a Project Record. The DFR presents the work undertaken and decisions taken during the project to achieve the overall project objective of developing a monoclonal antibody-based field test kit for blue-green algal toxins. It covers the work funded by NRA and further work undertaken by Biocode to repeat aspects of the project, this at their own cost. The Draft Final Report and final version, an R&D Note, should remain Externally Restricted due to a confidentiality agreement between NRA and Biocode.

Review criteria:

The project did not achieve its objective. However, in order for NRA to benefit from the project work, please consider the following review criteria.

1 Does the report present the full detail required for a summary document from the project ?

2 Of the information presented, is it clear to the NRA audience what was undertaken and achieved ?
What would you add/remove ?

3 Was the technical quality input by Biocode, as required for the development of a test kit of this type, good/average/poor ?
Qualify your comment by stating what Biocode's strengths were, or the aspects you would have changed.

4 What recommendations would you like to be added ?

5 Recommend dissemination of final R&D Note and Project Record

SUMMARY

In R and D project 348, Biocode Limited was contracted by the National Rivers Authority to develop and produce on site testing kits for the detection of microcystin-LR. This work required the development of novel monoclonal antibodies to microcystin-LR, the major hepatotoxin produced by blue green-algae.

Six monoclonal antibodies of the IgM subclass were prepared to microcystin-LR. Only one of these antibodies was shown to have an affinity to microcystin-LR by competitive ELISA. Immunoaffinity columns were prepared using this IgM antibody and the antibody was shown to be coupled to the support matrix. However, the immunoaffinity columns were shown not to bind detectable quantities of microcystin-LR. A number of factors were examined in an attempt to produce a useable immunoaffinity column:-

- method of antibody purification,
- an alternative gel matrix,
- method of application of the microcystin-LR,
- contact time between antibody and antigen,
- fragmentation of the IgM antibody into IgG-type fragments.

None of these led to the development of an immunoaffinity column able to bind detectable levels of microcystin-LR.

Further attempts at producing monoclonal antibodies to microcystin-LR led to the preparation of a further 24 hybridomas secreting antibodies to microcystin-LR. All these antibodies were of the IgM isotype. IgM antibodies are notoriously difficult to format into kits and few become commercialised. It is possible that the structure of microcystin-LR is such that only antibodies of the IgM isotype will be prepared to it. Alternatively, there are reports in the literature that microcystin-LR is immunosuppressive. In an attempt to circumvent this, Biocode limited launched an internally funded project to prepare monoclonal antibodies to ADDA.

ADDA, the portion of the microcystin-LR molecule that is unique, but common to most microcystins, was conjugated and used as an immunogen in mice. A polyclonal response was initiated with circulating antibodies able to bind conjugates of both microcystin-LR and ADDA. These circulating antibodies were primarily of the IgG isotype. However, no affinity for either microcystin-LR or ADDA could be demonstrated by competitive ELISA.

KEY WORDS

Microcystin-LR, ADDA , Monoclonal Antibody, IgM, Immunoaffinity column.

1 PROJECT DESCRIPTION

Blue-green algal (cyanobacterial) toxins have been demonstrated as being biologically active in a number of species, but their toxicity in humans is undefined and warrants more thorough investigation. Microcystin-LR is the major hepatotoxin produced by blue-green algae. It has a cyclic structure composed of seven amino acids, with a molecular weight of approximately 1000Da. Other structural variants exist that differ by two amino acids.

In R and D project 348 Biocode Limited was contracted by the National Rivers Authority to produce on-site testing kits using monoclonal antibodies. These kits would enable the easy, specific and rapid extraction and identification of microcystin-LR and some structurally related analogues. This work required the development of novel monoclonal antibodies. Work commenced on the project in October 1991.

Stage 1 of the project included the preparation of immunisation conjugates, the immunisation of mice to elicit a polyclonal immune response and the selection of suitable mice for monoclonal antibody production. The first half of Stage 2 encompassed the selection of hybridoma cells that secrete monoclonal antibodies that bind to microcystin-LR. This work was completed satisfactorily and was reported in detail in the interim reports of February, May, August and November 1992. Stage 3 of the project - production of at least 1g of antibody for future work - was also completed satisfactorily. The latter half of Stage 2 and Stage 4 - coupling of selected mooclonal antibodies to inert supports and preliminary evaluation of the immunoaffinity columns prepared - was also completed. Unfortunately the immunoaffinity columns were found to have no detectable capacity to bind microcystin-LR. This work was reported on in detail in the interim report of February 1993 In view of the results obtained in Stages 2 and 4, the project did not proceed to Stages 5. An overview of the work performed for R and D project 348 is presented here.

Biocode Limited undertook a second attempt at producing monoclonal antibodies to microcystin-LR, this time using a portion of the microcystin molecule (ADDA) as the immunogen. This work was conducted outside of the original project and funded by Biocode Limited. A brief summary of the outcome of the work is presented here.

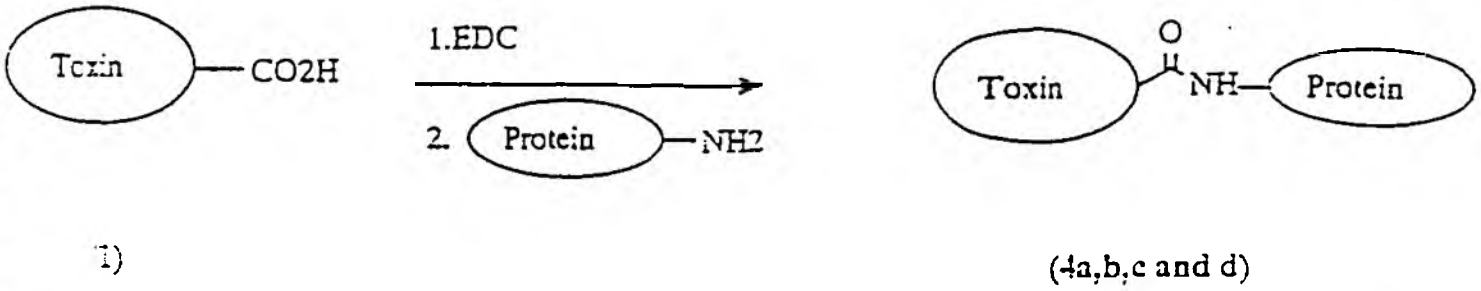
2 TECHNICAL SUMMARY

2.1 Summary of the work performed in R and D project 348.

2.1.1 Preparation of conjugates.

Four different conjugates of microcystin-LR were prepared. Two different carrier proteins were used - bovine serum albumin (BSA) and chicken gamma globulin (CGG). Each was conjugated to microcystin-LR with and without an ethyl diamine (EDA) linker group (Figure 2.1). Conjugates prepared using CGG as the carrier protein were used to immunise mice, whereas the conjugates prepared with the BSA carrier protein were used to coat ELISA plates and detect antibodies prepared to the immunogen.

Figure 2.1. Preparation of conjugates of microcystin-LR



1)

(4a,b,c and d)

a; Protein = BSA
b; Protein = EDA - BSA
c; Protein = C-ϒ-G
d; Protein = EDA - C-ϒ-G

2.1.2 Preparation of monoclonal antibodies to microcystin-LR

In order to determine the immune response of mice immunised with conjugates of microcystin-LR, serum samples were taken and assessed by ELISA. Sera were applied to ELISA plates coated with the microcystin-LR-EDA-BSA and microcystin-LR-BSA conjugates. Typical results are presented in Table 1. The same results were obtained with either of the plate coating conjugates. These results indicated a high level of circulating antibody in the sera of the immunised mice. The affinity of the serum antibodies for microcystin-LR was determined by competitive ELISA, where microcystin-LR was added in free solution in the ELISA with the serum antibody. No reduction in the binding of the serum antibody in the competitive ELISA was detected. Therefore, in order to determine the presence of serum antibodies that recognise microcystin-LR, ELISA plates were coated with unconjugated microcystin-LR. The serum antibodies gave a positive result in this assay (Table 2.1).

Strain of mouse	Immunogen	Maximum titre	
		Conjugate coated ELISA plates	Microcystin-LR coated ELISA plates
Balb/c x C57Bl6	Microcystin-LR-CGG	1/25000	1/1000
Balb/C	Microcystin-LR-CGG	1/25000	1/1000
Balb/C x C57Bl6	Microcystin-LR-EDA-CGG	1/25000	1/5000

Table 2.1. Assessment of the level of circulating antibodies in mice immunised with conjugates of microcystin-LR

Mice were selected for fusion on the basis of the level of circulating antibodies, determined by ELISA. Five mice were selected for fusion and preparation of monoclonal antibodies. From these fusions, a total of ten hybridoma cultures were prepared that were shown to contain antibody

detectable in ELISA using plates coated with conjugated microcystin-LR. The ability of the antibodies to bind to ELISA plates coated with unconjugated microcystin-LR was also determined. Of these, four were found to be producing antibody that bound nonspecifically in the ELISA. The results of ELISA analysis of the remaining six antibodies are presented in Table 2.2.

FUSION	CLONE	*ELISA units	
		Conjugate coated ELISA plate	Poly-L-lysine activated microcystin-LR coated ELISA plate
A005-1A	15C4	No positive result	1.46
A005-1C/E	9D5	0.431	No positive result
A005-1D	3D1	0.372	8.53
A005-1D	4B5	2.4	10.9
A005-1D	13C6	1.84	5.88
A005-1F	8D2	0.36	7.15

* Absorbance value in the ELISA (A_{450}) multiplied by the dilution of the antibody sample applied to the ELISA.

Table 2.2. Comparison of the binding of antibodies to the conjugated and unconjugated microcystin-LR in the ELISA

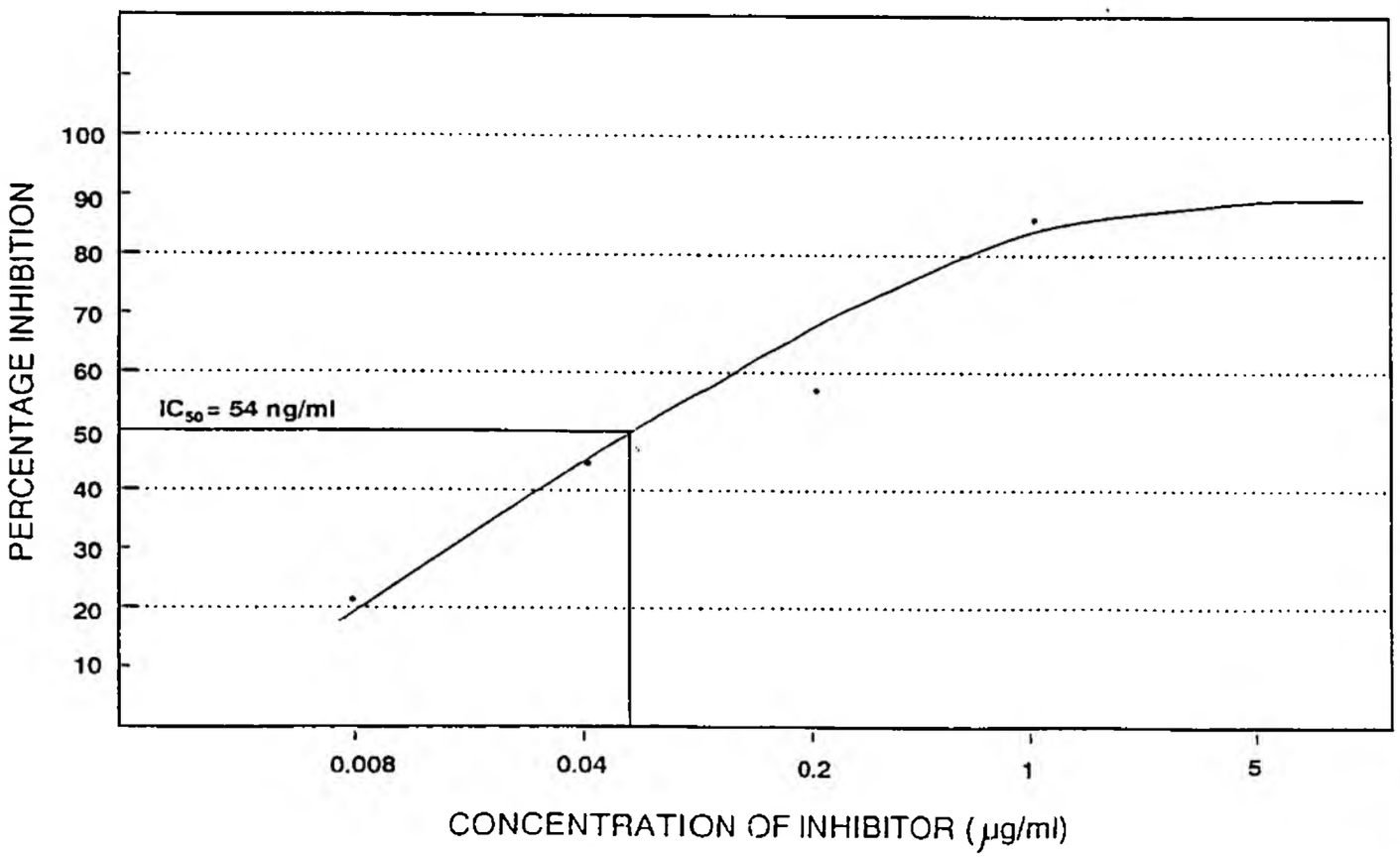
The affinity of these antibodies for microcystin-LR was determined by application to the competitive ELISA. These results are presented in Table 2.3. Affinity for microcystin-LR was only demonstrated reproducibly with the antibody A005-1D 13C6. The inhibiton curve is presented in Figure 2.2.

FUSION	CLONE	*IC_{50} (ng/ml)	
		Conjugate coated ELISA plate	Poly-L-lysine activated microcystin-LR coated ELISA plate
A005-1A	15C4	N/A	÷0.75
A005-1C/E	9D5	No inhibition	N/A
A005-1D	3D1	No inhibition	No inhibition
A005-1D	4B5	No inhibition	No inhibition
A005-1D	13C6	54	No inhibition
A005-1F	8D2	No inhibition	No inhibition

* concentration of microcystin-LR that reduces antibody binding by 50%.
÷ result obtained only once and found not to be reproducible.

Table 2.3. Assessment of the affinity of antibodies by competitive ELISA

Figure 2.2. Application of the antibody A005-1D 13C61G6 to the competitive ELISA



The subisotype of the antibodies was determined by application to an ELISA based subisotype test kit (Calbiochem). Each of them were shown to be of the IgM isotype.

The cell lines were cloned and monoclonal cell lines isolated. — more detail?

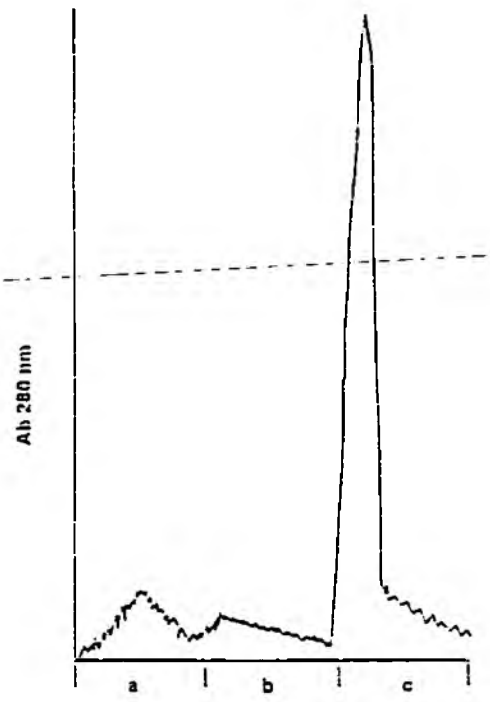
No stable monoclonal cell line was isolated from A005-1D 4B5. On the basis of the results presented in Table 2.3 formal frozen cell banks were established for the following cell lines. These banks have been assessed for the ability of the cells to retrieve from frozen and for *mycoplasma* contamination and have been shown to be satisfactory on both accounts.

A005-1A 15C49D5
A005-1D 13C61G6
A005-1F 8D21B5

2.1.3 Preparation of immunoaffinity columns using the A005-1D 13C61G6 antibody

In excess of 1g of the A005-1D 13C61G6 antibody was generated by hollow fibre fermentation. A sample of this antibody was purified by ammonium sulphate precipitation and the purity assessed by ion exchange chromatography (Figure 2.3).

Figure 2.3. Analysis of ammonium sulphate purified A005-1D 13C6 antibody by ion-exchange chromatography



The concentration of the purified A005-1D 13C61G6 antibody was assessed by ELISA. The antibody was coupled to cyanogen bromide activated Sepharose 4B at a number of different concentrations. The attachment of the antibody to the gel was demonstrated by the percentage coupling efficiency (the percentage reduction of protein concentration in the antibody solution after coupling, determined by absorption at 280nm - Table 2.4). At higher coupling concentrations, the sites on the gel become saturated leading to a reduction in the percentage coupling efficiency.

Antibody clone	Antibody coupling concentration (mg of antibody per ml of swollen gel)÷	Percentage coupling efficiency*
A005-1D 13C6	3.7 (5)	94.4
	7.5 (10)	80.8
	18.7 (25)	52.1

* as assessed by absorbance measurements at 280 nm.
÷ based on quantification ELISA figures. Values in brackets represent absorbance based coupling concentrations.

Table 2.4. The coupling efficiency of the A005-1D 13C61G6 antibody to cyanogen bromide activated Sepharose 4B

The gel coupled with the A005-1D 13C61G6 antibody was packed into Easi-Extract™ columns and assessed for its ability to bind microcystin-LR. A total of 10µg microcystin-LR was applied in solution to each of the columns. The following solvents were used to apply the microcystin-LR:-

phosphate buffered saline, pH7.4 (PBS)
borate/KCl buffer, pH8.5
10 and 20%v/v methanol in PBS
10 and 20% acetonitrile in PBS

The columns were washed with purified water to remove any non-bound material and the bound material was removed or eluted by the application of neat methanol. The eluate was analysed by HPLC using a method with a detection limit of 5ng. No microcystin-LR could be detected in the eluates. Analysis of the microcystin-LR in the solution before and after application to the column illustrated that the microcystin-LR passed straight through the column. In order to maximise antibody/antigen interactions the gel was incubated in free suspension for 1 hour at room temperature with microcystin-LR. Again, no binding of the microcystin-LR to the gel was demonstrated.

Finally, as an alternative, Triazine chloride activated Sepharose 4B gel was assessed as a support matrix. The Triazine chloride activated gel theoretically leads to a more stable binding of the antibody to the support matrix. Again, no binding of the microcystin-LR to the gel coupled with the A005-1D 13C61G6 antibody could be demonstrated.

In order to ensure that the best preparation of antibody was used for gel coupling six different methods of antibody purification were assessed. The method of purification of the A005-1D 13C61G6 antibody by PEG precipitation was chosen on the basis of recovering the least total protein and the most antibody that actively binds antigen (ie the purest preparation) (Table 2.5).

Coupling of the A005-1D 13C61G6 antibody purified by PEG precipitation to cyanogen bromide activated Sepharose 4B still led to no detectable binding of microcystin-LR.

Purification method	Percentage recovery		
	Total protein	Total antibody	Active antibody
Ammonium Sulphate precipitation	38	156	165
*Precipitation by PEG and ammonium sulphate	Not tested	3.1	1.1
Precipitation by PEG	13.8	105	135
Precipitation by dialysis with water	2.97	77	28
Ion exchange chromatography	46	112	96
Affi-T affinity chromatography	18	Not tested	6.44

* according to the method of Brooks et al, 1992 and A H Tatum, 1993

Table 2.5. Comparison of different methods of purification of the A005-1D 13C61G6 antibody

2.1.4 Preparation of IgG-type fragments of the A005-1D 13C61G6 IgM antibody

It was felt that the difficulties of incorporating the A005-1D 13C61G6 antibody into an immunoaffinity coloumn format was due primarily to the isotype of the antibody (IgM). Consequently an attempt was made to convert the IgM antibody into IgG-type fragments. Assessment of the purified A005-1D 13C61G6 antibody by HPLC size exclusion analysis indicated the presence of proteins of a mm of 1000KDa (equivalent to IgM antibody) but also the presence of contaminating proteins of other sizes. As the purity of the antibody preparation is essential for digestion work, an additional purification step was performed whereby the PEG precipitated antibody underwent large scale size exclusion chromatography. This material was then subjected to digestion by trypsinisation, followed by alkylation and reduction. Size exclusion analysis of the A005-1D 13C61G6 antibody before and after digestion illustrated a reduction in the proportion of molecules of a mm of 1000KDa (representative of IgM) and an increase in the proportion of molecules with a mm of 130KDa (representative of IgG), indicative of the generation of IgG-type fragments. Unfortunately, ELISA analysis of the A005-1D 13C61G6 antibody before and after digestion indicated a substantial loss in the antigen binding activity of the antibody after digestion. Subsequent coupling of the digested antibody to cyanogen bromide activated Sepharose 4B gel and application of microcystin-LR resulted in no detectable binding of microcystin-LR to the gel.

2.1.5 Preparation of further monoclonal antibodies to microcystin-LR

Further immunisations were performed using the conjugates of microcystin-LR prepared previously. Six fusions were performed on selected mice and from these a total of 24 cultures producing antibody detectable by ELISA were identified. All of these positives were shown to be of the IgM subclass.

2.2 Work performed independently by Biocode Limited

2.2.1 Preparation of monoclonal antibodies to ADDA

Conjugates were prepared of the ADDA portion of the microcystin molecule. As the EDA linker group had been used with some success in the preparation of conjugates of microcystin-LR, the following conjugates were prepared:-

ADDA-EDA-CGG
ADDA-EDA-BSA

Where the ADDA-EDA-BSA conjugate was used for immunisation, the ADDA-EDA-CGG conjugate was used for screening and *vice versa*. The results of the analysis of the antisera raised to the ADDA-EDA-BSA conjugate are presented in Table 2.6. The results from the analysis of the antisera to the ADDA-EDA-CGG were very similar. The ELISAs were performed using second antibodies specific to IgG and IgM and the results compared. No antibodies could be detected when the anti-IgM specific second antibody was used, suggesting that very few, if any, IgM antibodies were being produced.

Circulating antibodies in mice immunised with ADDA-EDA-BSA

Antigen used for testing	Maximum titre
ADDA-EDA-CGG	1/125000
microcystin-LR	No colour
Microcystin-LR-CGG	No colour
Microcystin-EDA-CGG	1/125000
EDA-CGG	1/25000

Table 2.6. Circulating antibodies in mice immunised with ADDA-EDA-BSA

No antibodies could be detected either to unconjugated microcystin-LR or microcystin conjugated directly to CGG. The results indicate that all the circulating antibodies in the antisera are to the EDA bridge group of the conjugates.

Further conjugates of ADDA were prepared this time using a direct coupling method. ADDA-ovalbumin conjugate was used for immunisation and the polyclonal antibodies were tested on ELISA plates coated with ADDA-BSA and microcystin-LR-BSA. The results are presented in Table 2.7. Although high levels of antibody appeared to be present, antibodies with an affinity for microcystin-LR or ADDA could not be demonstrated by competitive ELISA.

Circulating antibodies in mice immunised with ADDA-Ovalbumin

Antigen used for testing	Maximum titre
ADDA-BSA	1/25000
Microcystin-LR-BSA	1/125000

Table 2.7. Circulating antibodies in mice immunised with ADDA-ovalbumin

3 DISCUSSION

The conjugates prepared with the microcystin-LR were immunogenic in as much as they led to high levels of antibody in the mice that were immunised. However, no affinity for microcystin-LR could be demonstrated when the circulating antibodies were applied to a competitive ELISA. Some affinity for microcystin-LR was demonstrated by application of the sera in a direct ELISA to plates coated with the unconjugated microcystin-LR. The affinity was not measureable by this method.

The monoclonal antibodies prepared to the conjugated microcystin-LR were selected on their ability to recognise both the conjugated and unconjugated forms of microcystin-LR applied to ELISA plates. One of these antibodies was shown to have a high affinity for microcytin-LR that was measureable by competitive ELISA (A005-1D 13C61G6). This suggests that the other monoclonal antibodies had a low affinity for microcystin-LR.

A total of 11 fusions were performed on mice immunised with conjugates of microcystin-LR. All the antibody producing hybridomas that were tested and shown to produce antibodies to microcystin-LR were shown to be secreting antibodies of the IgM isotype. Some immunogens (often polymeric compounds) result in an IgM response only. It appears from these results that immunisations with microcystin-LR led to an IgM response in mice. This may be due either to the structure of the compound and the particular immunological response to it, or to the immunosuppressive effects of microcystin-LR cited in the literature.

The A005-1D 13C61G6 antibody was purified, coupled to a gel matrix and the gel packed into immunoaffinity columns. The purity and antigen binding activity of the antibody was determined prior to coupling and was shown to be satisfactory. The purification procedure was examined carefully as IgM antibodies are particularly unstable in solution and consequently difficult to purify. Assessment of the total protein concentration of the antibody solution before and after coupling showed that the antibody did bind to the gel. However, no binding of the microcystin-LR antigen to the antibody coupled gel could be illustrated. The HPLC method used to detect the microcystin-LR has a minimum detection limit of 5ng.

The A005-1D 13C61G6 antibody had been selected on the basis of its ability to perform in ELISA. This procedure utilises antibody/antigen binding times of approximately one hour at room temperature. In order to mimic the antigen binding time of an ELISA the coupled gel was incubated in free suspension with the microcystin-LR antigen at room temperature for one hour. This still resulted in no detectable antigen binding. This suggests that during the coupling process the ability of the antibody to end antigen has been lost. The stability of the bonding of the antibody to the gel was improved by use of Triazine activated Sepharose. Finally the solvent used to apply the antigen to the coupled antibody was examined. Either of these measures led to the binding of detectable levels of microcystin-LR to the antibody coupled gel.

The A005-1D 13C61G6 antibody is the first IgM antibody that we have used to prepare immunoaffinity columns at Biocode. IgM antibodies are notoriously difficult to use in kit formatting and few become commercialised. Attempts were made to prepare IgG-type fragments from the IgM antibody. Assessment of the sizes of the protein molecules present and their relative concentrations verified the conversion of the IgM antibody into molecules of equivalent size to IgG. However, the procedure of trypsinisation, alkylation and reduction led to a loss in the antigen

binding activity of the antibody. Subsequent coupling of the digested antibody to a gel support matrix led to the preparation of immuoaffinity columns that were unable to bind detectable levels of microcystin-LR.

The polyclonal response that is elicited in response to an immunogen is dependent on the structure of the antigen molecule as a whole. With this in mind, a portion of the microcystin-LR molecule - ADDA - was used to prepare conjugates for immunisation. ADDA is structurally unique to microcystins, but common to most of them. It was hoped that presentation of this portion of microcystin-LR in the absence of the rest of the molecule would ilicit a different immunological response, hopefully primarily of the IgG type. In the first instance, conjugates of ADDA were prepared using the EDA bridge group. Although this had been used sucessfully with the microcystin-LR immunisations, in this instance it was found that most of the antibodies in the polyclonal response were to the EDA bridge rather than the ADDA hapten. This implies that the EDA bridge was the most immunogenic part of the conjugate. Further conjugates were then prepared with a more direct linkage. Here, high titres of IgG polyclonal antibodies were produced in response to the immunisations. As found with previous immunisations with the conjugates of microcystin-LR, no affinity for either ADDA or microcystin-LR could be demonstrated by competitive ELISA. This suggests that few, if any, high affinity antibodies were present.

4 CONCLUSIONS

Immunisation of conjugates of microcystin-LR led to the development of high levels of polyclonal antibodies that were primarily of the IgM type. From these immunisations, one IgM monoclonal antibody was prepared to microcystin-LR that had demonstrable affinity in the competitive ELISA. Attempts at utilising this antibody to produce an immunoaffinity column able to bind detectable levels of microcystin-LR were unsuccessful.

Immunisations with conjugates of a portion of the microcystin-LR molecule - ADDA - led to the development of high levels of circulating antibodies of the IgG type. However, the affinity of the antibodies for microcystin-LR could not be demonstrated.

At this point Biocode concluded that it was unlikely that a high affinity monoclonal antibody could be prepared to microcystin-LR from animals exhibiting such a polyclonal response.