R&D Project 348

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

Biocode Ltd
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R&D Draft Final Report 348/7/A
(R&D Project Record)

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

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R&D Draft Final Report 348/7/A

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Appendices

Interim Progress Report **R&D Project 348**

Development of A Field Test Kit For Detection of Blue-Green Algal Toxins

Biocode Limited
February 1992
R&D 348/01/A

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SUMMARY

Work has commenced on Stage 1 of this project to develop monoclonal antibodies to microcystin LR (research work commenced on the 8th November 1991). To date three immunisation conjugates of microcystin-LR have been prepared (3a, 3b and 4c) along with two microcystin-LR conjugates (4a and b) for coating of ELISA plates. Balb/C and Balb/CX C57B1 (F1) hybrid mice have received three inoculations and serum samples obtained for antibody quantification (Stage 1.2.1). Initial analysis of the circulating antibodies by ELISA (Stage 1.2.2) show that antibodies against microcystin-LR are present , albeit at low titres. Difficulty has been experienced at obtaining sufficient microcystin-LR for immunisation and ELISA plate coating purposes.

KEY WORDS

Microcystin-LR, Conjugation, Immunisation, ELISA, Antibody.

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.

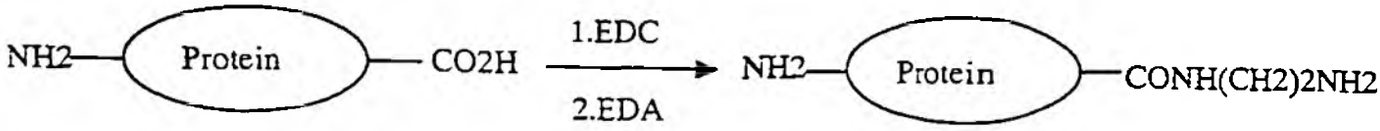
Biocode Limited is in the process of developing on-site testing kits using monoclonal antibodies. These kits are to enable the easy, specific and rapid extraction and identification of microcystin-LR and some structurally related analogues. This work requires the development of novel monoclonal antibodies. Stage 1 of the project includes 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 latter part of Stage 1 encompasses the production and selection of hybridoma cells that secrete monoclonal antibodies. Selection of mice for this procedure is based on the analysis of the level and affinity of circulating serum antibodies in the immunised mice using the enzyme linked immuno sorbent assay (ELISA). This assay is based on the specific attachment of antibodies to ELISA plates coated with conjugated microcystin-LR. The affinity of this attachment is then assessed by inhibition ELISA.

2 REVIEW OF TECHNICAL PROGRESS FOR OCTOBER 1991 - JANUARY 1992

2.1 Preparation of at least two conjugates for testing (Stage 1.1)

Two different methods were explored for direct and indirect linkage of the toxin to the proteins bovine serum albumin (BSA),chicken-gamma-globulin (C-ɣ-G) and also with the ethylene diamine modified BSA and C-ɣ-G (EDA-BSA and EDA-C-ɣ-G respectively), as described by F. S. Chu, acting as the bridged conjugates (see Scheme 1)

SCHEME 1



(1) (2a,b)

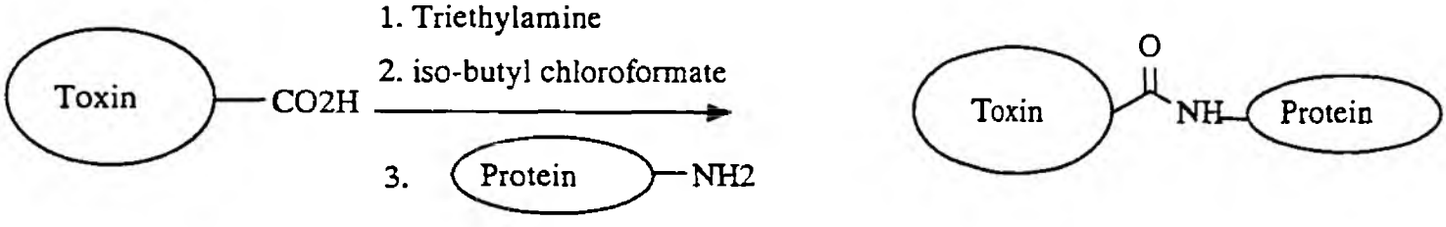
a; Protein = BSA

b; Protein = C-ɣ-G

2.1.1 The mixed anhydride method

Typically 5mg of toxin was solubilised in tetrahydrofuran (THF) prior to treatment with triethylamine and iso-butyl chlorformate at -5°C. The preformed mixed anhydride was then added slowly to the appropriate protein solution in aqueous pyridine. Purification was achieved via dialysis against purified water (see Scheme 2).

SCHEME 2



(1) (3a,b)

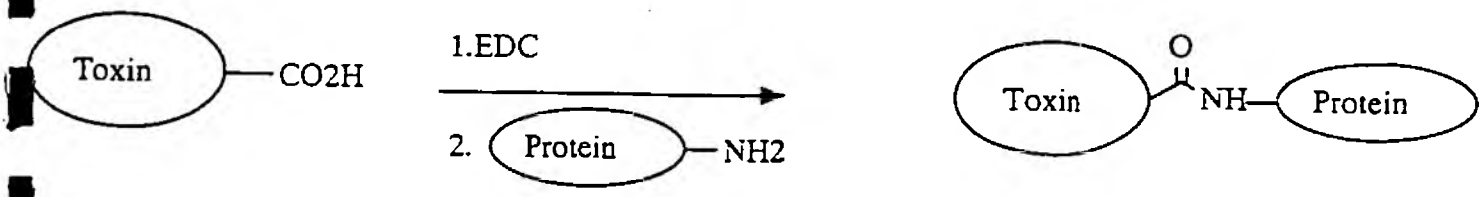
a; Protein = C-ɣ-G

b; Protein = EDA - C-ɣ-G

2.1.2 Carbodiimide coupling

Initially the toxin was taken up in methanol prior to dilution with purified water. This solution was added to the appropriate aqueous protein followed by the carbodiimide coupling reagent. The pH was maintained at 5 via addition of sufficient 0.2M hydrochloric acid over a period of 5 hours. Purification was achieved by chromatography using a Sephadex PD-10 column. The protein fraction was then lyophilised to yield the desired conjugates (see Scheme 3.)

SCHEME 3



(1)

(4a,b,c and d)

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

2.2 Production of monoclonal antibodies to conjugates (Stage 1.2)

2.2.1 Immunisation

Mice were immunised with each of the immunisation conjugates prepared in Stage 1.1 above.

The appropriate immunisation conjugate was solubilised in phosphate buffered saline (PBS), pH 7.4, to a concentration of 2mg/ml. This was emulsified with an equal volume of Freund's adjuvant. Two strains of mice were immunised, pure bred Balb/C and Balb/CX C57Bl/6 crossbred mice. All mice were immunised in batches of 1 to 8, and each individual received a series of intraperitoneal inoculations. For each immunisation, the F1 hybrid mice received 300ul of inoculum, initially prepared using Freund's complete adjuvant. All subsequent immunisations were of the same volume and route, but prepared with Freund's incomplete adjuvant. The pure bred Balb/C mice were immunised with a series of 100ul inoculations, all prepared with Freund's incomplete adjuvant.

Serum samples were prepared from blood taken from the suborbital vein. The blood sample (approximately 500ul) was allowed to clot at 2-8°C overnight and the serum isolated by centrifugation.

2.2.2 Polyclonal screening

The circulating antibody in the immunised mice was assessed by applying serum samples to the ELISA.

ELISA plates were coated with either of the two plate coating conjugates prepared in Stage 1.1. Serum from each mouse was tested on both plate coating conjugates. Serum samples were applied to the plates in a series of dilutions (ie titrated) and the maximum dilution that gave a detectable reading (>0.2), at absorbance value (A_{450}), in the assay, was determined. The affinity of the antibodies for microcystin-LR was assessed by inhibition ELISA. A suboptimal concentration of serum (selected from the titration ELISA) was applied to the ELISA plates in the presence of the inhibitor, in this case microcystin-LR. The microcystin-LR was applied to the ELISA in PBS, pH 7.4 in a series of different concentrations (1ng/ml to 100ug/ml). The concentration of microcystin-LR required to reduce the binding of the antibodies to the ELISA plate by 50%, was assessed.

3 INTERIM RESULTS, DISCUSSION AND CONCLUSIONS

3.1 Preparation of at least two conjugates for testing (Stage 1.1)

Initially the mixed anhydride method was used for the preparation of the direct and bridged C-Y-G immunogens (3a and b). Unfortunately the yields (ca. 2mg) were too low and an alternative method of production had to be employed. The toxin and proteins were coupled in the presence of a water soluble carbodiimide. They were purified using a Sephadex PD-10 column. Upon lyophilisation the desired conjugates (4a-d) were obtained in favourable yields.

3.2 Production of monoclonal antibodies to conjugates (Stage 1.2)

3.2.1 Immunisation

Mice were immunised with each of the immunisation conjugates prepared in Stage 1.1 above. The scheme used for the immunisation and sampling of sera is summarised in Table 3.1:

PROCEDURE	ADJUVANT	TIME (DAYS)
1st immunisation	Freund's Complete (F1 hybrids only)	0
2nd immunisation	Freund's Incomplete	21
1st serum sample		35
3rd immunisation	Freund's Incomplete	42
2nd serum sample		56

TABLE 3.1: Scheme for the immunisation of mice with conjugates of microcystin-LR.

3.2.2 Polyclonal screening

Table 3.2 summarises the number and strain of mice that were immunised with each of the conjugates. The number of mice and different strains immunised with each immunogen was limited by the quantity of conjugate available, owing to shortages of microcystin-LR. A positive mouse was defined as giving an A_{450} greater than 0.2 in the ELISA when the serum was diluted by 1/1000 or more. The maximum titre represents the maximum dilution of antibody that gave an A_{450} greater than 0.2 in the ELISA. Results obtained using the two plate coating conjugates were the same for each case.

STRAIN OF MOUSE	IMMUNOGEN	NO OF MICE IN BATCH	NO +VE MICE	MAXIMUM TITRE
Balb/C X C57Bl/6	3a	5	3	1/5000
Balb/C X C57Bl/6	4c	4	4	1/25000
Balb/C	4c	8	8	1/25000
Balb/C X C57Bl/6	3b	1	1	1/25000

TABLE 3.2 Assessment of the level of circulating antibody in mice immunised with conjugates of microcystin-LR.

The maximum titre represents the best result obtained from a serum sample taken from one mouse in the batch prepared by the mixed anhydride route (see section 2.1 Scheme 2).

In order to assess the affinity of the circulating antibodies in the mice, sera that had a maximum titre of 1/5000 or more were used in the inhibition ELISA. Each serum sample was diluted to a level which would give a suboptimal A_{450} in the ELISA (0.4 to 0.6). In the first instance, microcystin-LR was used as the inhibitor and sera were assessed on both types of ELISA plate coating conjugates. Application of as much as 100ug/ml of microcystin-LR was found not to affect the binding of suboptimal quantities of serum antibodies to the ELISA plates.

In order to establish that the antibodies had some affinity for the conjugates prepared in Stage 1.1, selected serum samples were analyzed in a further inhibition ELISA, this time using the appropriate immunisation or plate coating conjugates. The results are summarised in Table 3.3. With one exception, it was found that good inhibition could be achieved using either the plate coating conjugate or the immunisation conjugate as the inhibitor.

MOUSE STRAIN	IMMUNISATION CONJUGATE	ELISA PLATE COATING CONJUGATE	INHIBITOR	IC50 (ug/ml)
Balb/C X C57Bl/6	4c	4a	4a	0.8
			4c	None
		4b	4b	0.3
			4c	None
Balb/C	4c	4a	4a	50.0
			4c	60.0
		4b	4b	1.0
			4c	13.0
Balb/C X C57Bl/6	3b	4a	4a	0.6
		4b	4b	0.5

TABLE 3.3 Serum inhibition ELISA using immunisation or ELISA plate coating conjugates as inhibitors.

The bridged conjugate (3b) was not used as an inhibitor as it was in short supply.

To establish whether the circulating antibodies have any affinity for unconjugated microcystin-LR, a method was devised for the coating of ELISA plates with the unconjugated toxin. Plates were coated with solutions of microcystin-LR ranging from 50ng to 50ug/ml. Sera from each of the immunised mice were tested and only one gave a positive result (A450>0.2). This mouse was the single mouse that had been immunised with the 3b immunisation conjugate. In order to detect circulating antibodies to microcystin-LR, the ELISA plates had to be coated with no less than 50ug/ml of the toxin.

3.3 DISCUSSION

The titration ELISA results illustrate that the mice have responded highly to the immunisation conjugates. The protein used to prepare the immunisation conjugates differs from the protein used to prepare the plate coating conjugates. Therefore, it is unlikely that the antibodies detected in the ELISA are binding to the carrier protein, but are most likely to be specific to the conjugated microcystin-LR that is shared by the immunogen and the plate coating conjugate.

Results obtained from inhibition ELISAs suggest that the antibodies detected plate coating conjugates used in the original ELISA procedure have a poor affinity for microcystin-LR. It is possible that conjugation of the microcystin-LR to protein, during the preparation of conjugates, sterically alters the arrangement of the microcystin-LR molecule.

3.4 CONCLUSIONS

The microcystin-LR presented on the carrier protein bears only a partial resemblance to free microcystin-LR. Therefore, many of the antibodies produced in the mouse will have an affinity for the conjugated microcystin-LR and fewer will have an affinity for the free microcystin-LR. The plate coating conjugates will also have a microcystin-LR derivative that has been altered in the same manner as the immunogen. Use of such a conjugate in ELISA screening will selectively detect antibodies with a high affinity for the conjugated, rather than the free, microcystin-LR. This is borne out by the high affinity of the antibodies for the immunisation and plate coating conjugates when these were used as inhibitors in the inhibition ELISA in place of microcystin-LR.

3.5 RECOMMENDATIONS

Application of serum from the mouse immunised with the bridged conjugate (3b) to plates coated with unconjugated microcystin-LR shows that we can produce antibodies with an affinity for the free toxin. However, in order to increase the number of mice for future fusions it is important that more of the bridged conjugate (3b) is prepared. It is possible that a spatial separation of the microcystin-LR from the carrier protein minimises the steric alteration of the toxin once conjugated.

In order to make appropriate selections of mice for future fusion and monoclonal antibody production, it is important that sera are screened using plates coated with the unconjugated microcystin-LR. This means that the requirements for microcystin-LR are substantially greater than first estimated.

4 COST OF THE WORK CARRIED OUT IN THE PERIOD OCTOBER 1991 - JANUARY 1992

During the first three month period of the project (14 October 1991 - 15 January 1992), the total cost of work carried out is given below and related to the 1991/1992 and total project budgets.

	This Period	1991/92 Total	Project Total
	£	£	£
Staff salaries	15 800	29 000	67 000
Travel & Subsistence	275	500	1 500
Consumables (+ minor capital items)	8 180	15 000	30 000
Reports	275	500	1 500
Total	24 530	45 000	100 000

5 ESTIMATE OF THE COST OF WORK FOR THE PERIOD JANUARY 1992 - APRIL 1992

It is estimated that the cost of the work to be carried out in the next period (until the end of March 1992) will be as budgeted by the NRA.

	£
Staff salaries	13 200
Travel & subsistence	225
Consumables (+ minor capital items)	6 820
Reports	225

Total	20 470

6 PROGRAMME OF WORK FOR THE PERIOD JANUARY 1992 - APRIL 1992

During the next period until the end of March 1992, the project is expected to proceed to plan *i.e.* towards the completion of Stage 1.

Further immunisations and subsequent polyclonal screenings will be carried out followed by fusions and subsequent cloning of the chosen antibodies. Full details are given in the original Biocode Project Proposal (contained in the NRA Research Contract Document).

7 REVIEW OF FACTORS LIKELY TO AFFECT COMPLETION OF THE WORK PROGRAMME

The project is proceeding to plan and there are no known factors at present other than possible shortages of highly purified microcystin-LR which are likely to affect the successful conclusion of the project on schedule.

Interim Progress Report **R&D Project 348**

Development of A Field Test Kit For Detection of Blue-Green Algal Toxins

Biocode Limited
May 1992
R&D 348/02/A

20/05/92

CONTENTS

SUMMARY

Work has continued on Stage 1 of this project to develop monoclonal antibodies to microcystin-LR. A single mouse was selected for fusion on the basis of the results presented in the previous report. From this, three cultures have been selected that bind to microcystin-LR coated plates. The isolation and characterisation of monoclonal cell lines from these cultures is ongoing.

Initial difficulties in detecting antibodies that bind to the unconjugated microcystin-LR have been overcome by the use of enzyme-linked immuno sorbent assay (ELISA) plates coated directly with the unconjugated toxin. The quantity of microcystin-LR required for plate coating has been substantially reduced by pretreating the ELISA plates with poly-L-lysine.

Immunisations with the unbridged microcystin-LR conjugate have continued. In addition a new batch of bridged conjugate has been prepared and used to immunise a further batch of mice. As a result, another four mice have been selected for fusion. From these four fusions fourteen additional cultures have been isolated that produce antibody to microcystin-LR. Monoclonal cell lines are currently being selected from these cultures.

KEY WORDS

Microcystin-LR, ELISA, Fusion, Monoclonal Antibody.

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.

Biocode Limited is in the process of developing on-site testing kits using monoclonal antibodies. These kits are to enable the easy, specific and rapid extraction and identification of microcystin-LR and some structurally related analogues. This work requires the development of novel monoclonal antibodies. Stage 1 of the project includes 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 latter part of Stage 1 encompasses the production and selection of hybridoma cells that secrete monoclonal antibodies specific to microcystin-LR and its analogues. Selection of mice for this procedure is based on the analysis of the level and affinity of circulating serum antibodies in the immunised mice using the enzyme linked immuno sorbent assay (ELISA). This assay is based on the specific attachment of antibodies to ELISA plates coated with conjugated or free microcystin-LR. The affinity of this attachment is then assessed by inhibition ELISA.

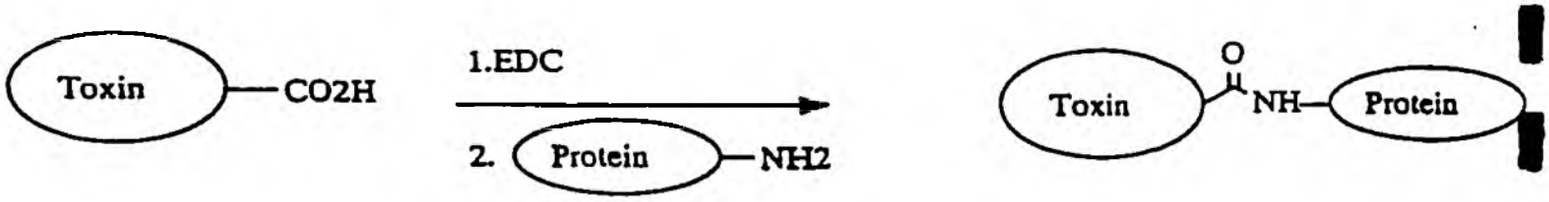
2.1 Preparation of at least two conjugates for testing (Stage 1.1)

2.1.1 Preparation of a new batch of the bridged immunisation conjugate

The preparation of this conjugate is summarised in Scheme 1.

Initially, the toxin was dissolved in methanol, prior to dilution with purified water. This solution was then added to an aqueous solution of the ethylene diamine modified chicken gamma globulin (CGG) followed by the carbodiimide coupling reagent. The pH was maintained at 5 via addition of sufficient 0.2M hydrochloric acid over a period of 5 hours. Purification of the conjugate was achieved by chromatography using a Sephadex PD-10 column. The protein fraction was then lyophilised to yield the desired immunogen (4d).

SCHEME 1

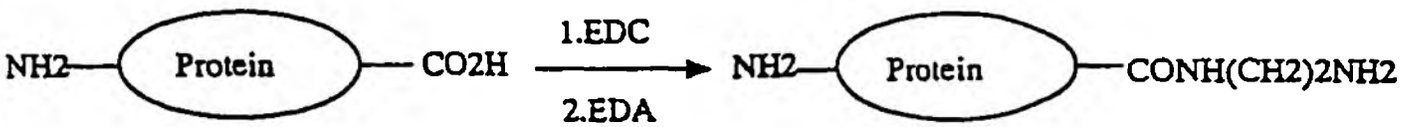


(1) (4a,b,c and d)

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

Schemes 2 and 3 illustrate the preparation of other conjugates, which have been used for the continued immunisation of mice initiated in R&D 348/01/A. The details of the preparation of these conjugates was presented in R&D 348/01/A.

SCHEME 2

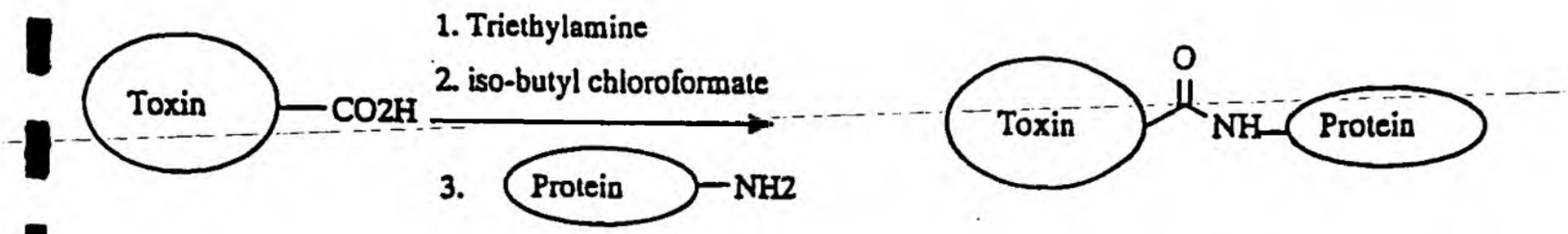


(1) (2a,b)

a; Protein = BSA

b; Protein = C-Y-G

SCHEME 3



(1) (3a,b)

a; Protein = C-Y-G

b; Protein = EDA - C-Y-G

2.2 Production of monoclonal antibodies to conjugates (Stage 1.2)

2.2.1 Immunisation

The schedule used for the immunisation of all mice is summarised in Table 2.1:-

PROCEDURE	ADJUVANT	TIME (DAYS)
1st immunisation	Freund's Complete (F1 hybrids only)	0
2nd immunisation	Freund's Incomplete	21
1st serum sample		35
3rd immunisation	Freund's Incomplete	42
2nd serum sample		56

TABLE 2.1 Scheme for the immunisation of mice with conjugates of microcystin-LR

2.2.2 Development of an ELISA method which selectively detects antibodies which bind to unconjugated microcystin-LR

In an attempt to reduce the quantities of microcystin-LR required in the ELISA, three different activation methods were assessed for their ability to improve the binding of microcystin-LR to the ELISA plates. ELISA plates were pretreated in each of the following ways:

i) 0.2% v/v glutaraldehyde solution for 90 minutes at room temperature,
ii) 1mg/ml solution of poly-L-lysine for 15 minutes at room temperature,
iii) 1% w/v protamine sulphate solution for 90 minutes at room temperature.

The plates were then washed with purified water and coated with different concentrations of microcystin-LR. The different coating methods were assessed by the application of sera sampled from an immunised mouse.

2.2.3 Polyclonal screening

The circulating antibody in the immunised mice was assessed by applying serum samples to the ELISA.

Serum from each mouse was tested on ELISA plates coated with unconjugated microcystin-LR. Serum samples were applied to the plates in a series of dilutions (ie titrated). The

maximum dilution that gave a reading, or absorbance value (A_{450}), of more than 0.2, was determined.

2.2.4 Fusion

Three days prior to the fusion, the mouse received an intravenous (i/v) inoculation into the tail vein with a solution of 100μg of the immunisation conjugate in phosphate buffered saline (PBS) pH 7.4. The spleen was removed on the day of the fusion and the splenocytes suspended in GKN medium with the myeloma cell line P3X63Ag8.6.5.3. The splenocytes were added to the myeloma cells at a ratio of 20:1 and pelleted by centrifugation. The cell mixture was fused by the addition of a 50%v/v solution of GC grade polyethylene glycol (PEG). The fusion product was resuspended in hypoxanthine/aminopterin/thymidine (HAT) medium, which is selective for the growth of hybridoma cells.

2.2.5 Fusion Screen

After incubation at 37°C, 5% v/v CO_2, for 14 to 21 days, samples of media were taken from each of the 480 cultures arising from one fusion. These samples were applied to ELISA plates in duplicate wells, coated with unconjugated microcystin-LR. Samples which gave an (A_{450}) greater than 0.2, were selected as positive.

3 INTERIM RESULTS, DISCUSSION AND CONCLUSIONS

3.1 Preparation of at least two conjugates for testing (Stage 1.1)

3.1.1 Preparation of a new batch of the bridged immunisation conjugate

The polyclonal response of the mice immunised with this conjugate is presented in Table 3.2, (Section 3.2.3). These results indicate that the conjugate was prepared successfully.

3.2 Production of monoclonal antibodies to conjugates (Stage 1.2)

3.2.1 Immunisation

A new batch of Balb/C X C57Bl/6 hybrid mice received two successive inoculations with the newly prepared bridged immunisation conjugate (4d) prepared in 3.1.1. above. Two weeks after the second inoculation, sera were sampled for polyclonal screening (Section 3.2.3).

All existing mice, immunised with conjugates 3a, 3b and 4c (detailed in R&D 348/01/A) received a third inoculation of the appropriate conjugate. Two weeks after inoculation, serum samples were taken for polyclonal screening (Section 3.2.3.).

3.2.2 Development of an ELISA method which selectively detects antibodies which bind to unconjugated microcystin-LR

ELISA plates were activated by the three different methods detailed in Section 2.2.2. Each of these plates were then incubated with different concentrations of the microcystin-LR. Serum from a single mouse immunised with the first batch of bridged immunisation conjugate (3b) was diluted and applied to the activated ELISA plates at each antigen coating level. The minimum antigen coating level that gave an A_{450} of more than 0.2 by the application of the serum, for each of the ELISA plate activation methods, is summarised in Table 3.1.

METHOD OF ELISA PLATE ACTIVATION	OPTIMUM ANTIGEN COATING LEVEL (μg/ml)
No activation	50μg/ml
0.2 v/v glutaraldehyde	10μg/ml
1% w/v protamine sulphate	10μg/ml
1mg/ml poly-L-lysine	2μg/ml

TABLE 3.1 Comparison of the different methods of ELISA plate activation

3.2.3 Polyclonal screening

The results of ELISA analysis of serum sample are summarised in Table 3.2. Serum samples were taken after three immunisations, with the exception of the last batch of mice listed,

which were sampled after two immunisations with the newly prepared batch of the 3b immunogen. The results quoted are the maximum titres obtained from an individual mouse in that particular batch. The maximum titre represents the maximum dilution of antibody that gave an A_{450} greater than 0.2 in the ELISA.

A direct comparison is made between the titres obtained using plates coated with the two plate coating conjugates (4a) and (4b), which for all but one instance, were the same, and titres obtained on ELISA plates coated with microcystin-LR. The latter were coated at a level of 5μg/ml on poly-L-lysine activated plates. It is clear from the results that although many of the circulating antibodies bind to the plate coating conjugates 4a and 4b, only a fraction of these appear to bind to the unconjugated microcystin-LR. It was also found that increasing the coating level of unconjugated microcystin-LR on the ELISA plates did not increase the titres of the sera, hence the level of applied antigen is not a limiting factor in the ELISA (results not shown).

STRAIN OF MOUSE	IMMUNOGEN	MAXIMUM TITRE	
		Conjugate coated ELISA plates.	Microcystin-LR coated ELISA plates.
Balb/C X C57Bl/6	3a	1/5000	1/1000
Balb/C X C57Bl/6	4c	1/25000	1/1000
Balb/C	4c	1/25000	1/1000
Balb/C X C57Bl/6	3b	1/25000	1/5000
Balb/C X C5Bl/6	4d (new batch)	1/25000 for 4a 1/125000 for 4b	1/1000

TABLE 3.2 Assessment of the level of circulating antibody in mice immunised with conjugates of microcystin-LR

3.2.4 Fusion

On the basis of results obtained from the analysis of polyclonal sera, mentioned in R&D 348/01/A, a single mouse was selected for an early fusion. A Balb/C X C57Bl/6 mouse was selected and this had received two immunisations with the unbridged immunisation conjugate (4c). The fusion of the spleen from this mouse was performed successfully (Table 3.3).

In addition, the single Balb/C X C57Bl/6 mouse that had received two immunisations of the

first batch of bridged immunisation conjugate (3b) was selected for fusion. Unfortunately, after the i/v administration of the conjugate, the mouse died. Post mortem examination revealed no evidence of the toxic effects of microcystin-LR.

The next fusion was performed on a single mouse from the batch of Balb/C X C57Bl/6 mice that had been immunised with the new batch of bridged immunisation conjugate (4d), prepared in Section 3.1.1.

Furthermore, after administration of the third inoculation of the unbridged immunisation conjugate (4c), two Balb/C X C57Bl/6 mice and a single Balb/C mouse were also selected for fusion.

The results of these fusions are summarised in Table 3.3. The number of splenocytes isolated from each spleen indicates the extent of the response of the mouse to immunisation. The number of hybridoma clones arising from the fusion of these splenocytes indicates the success of the fusion.

FUSION NUMBER	STRAIN OF MOUSE	IMMUNOGEN	No. OF SPLENOCYTES	No. OF HYBRIDOMA CLONES
A005-1A	Balb/C X C57Bl/6	4c	1.7×10^8	650
A005-1C/E (2 spleens used)	Balb/C X C57Bl/6	4c	3.2×10^8	546
A005-1D	Balb/C	4c	6.1×10^8	1454
A005-1F	Balb/C X C57Bl/6	4d	3.0×10^8	239

TABLE 3.3 Summary of the number of splenocytes and hybridomas yielded from the five mice selected for fusion

3.2.5 Fusion Screen

Supernatants from each of the 480 culture wells from five fusions, were tested by ELISA on poly-L-lysine activated plates coated with unconjugated microcystin-LR.

The number of fusion wells found to contain antibody to microcystin-LR are summarised in Table 3.4.

FUSION NUMBER	MOUSE STRAIN	IMMUNOGEN	No OF CULTURE WELLS CONTAINING HYBRIDOMAS PRODUCING ANTIBODY TO MICROCYSTIN-LR
A005-1B	Balb/C X C57Bl/6	4c	3
A005-1C/E (2 spleens used)	Balb/C X C57Bl/6	4c	4
A005-1D	Balb/C	4c	9
A005-1F	Balb/C X C57Bl/6	4d	1

TABLE 3.4 Summary of the number of hybridomas producing antibody to microcystin-LR selected from the five fusions

These cultures are currently undergoing cloning by limiting dilution.

3.3 DISCUSSION

Polyclonal responses of batches of immunised mice that were initiated in the R&D 348/01/A report indicate that the mice have continued to respond highly to the immunisation conjugates. The new batch of mice immunised with the freshly prepared bridged immunisation conjugate (4d) have also responded well.

Pre-activation of ELISA plates with poly-L-lysine has reduced the requirements of microcystin-LR necessary for the preparation of plates coated with the unconjugated toxin. However, the maximum titration of polyclonal sera on these plates was substantially less than when the same samples were applied to conjugate coated ELISA plates. Preparation of ELISA plates coated with higher concentrations of microcystin-LR did not increase the maximum titration, indicating that the microcystin-LR was not limiting. This suggests that only a fraction of the circulating antibody binds to unconjugated microcystin-LR. This possibility is also indicated by the small number of hybridomas that secrete antibodies that are detectable by using the unconjugated microcystin-LR coated ELISA plates, despite the fact that many hundreds of hybridoma clones were produced.

3.4 CONCLUSIONS

It was concluded in the previous report that the microcystin-LR presented on the carrier protein bears only a partial resemblance to free microcystin-LR. Thus, many of the antibodies produced in the mouse will have an affinity for the conjugated microcystin-LR and fewer will have an affinity for the free microcystin-LR. Results presented in this report indicate that only a small proportion of the hybridomas produced from the fusions secrete antibody with an affinity for microcystin-LR. This reinforces the conclusion made in the R&D 348/01/A report.

However, continued use of the microcystin-LR coated ELISA plates has led to the selection of a total of 17 hybridoma cultures that secrete antibody to microcystin-LR. In order to obtain sufficient cell lines for further analysis a total of five fusions have been performed. This is in excess of the requirements of any other similar project performed at Biocode to date.

3.5 RECOMMENDATIONS

Efforts are to be concentrated on the selection of monoclonal cell lines from the 17 cultures that produce antibody to microcystin-LR. Once monoclonal lines have been established, frozen stocks of the cells are to be set up in liquid nitrogen. Work will also commence on the evaluation of the affinity of the antibodies for microcystin-LR. The stability of the relevant cell lines in culture and their antibody productivity will also be determined. On the basis of these results up to five cell lines will be selected for antibody production and evaluation of the coupling of the antibody to a support matrix.

4 COST OF THE WORK CARRIED OUT IN THE PERIOD JANUARY 1992 - APRIL 1992

During the second three month period of the project (15 January 1992 - 5 April 1992), the total cost of work carried out is given below and related to the 1991/1992 and total project budgets.

	This Period	1991/92 Total	Project Total
	£	£	£
Staff salaries	13 200	29 000	67 000
Travel & Subsistence	225	500	1 500
Consumables (+ minor capital items)	6 820	15 000	30 000
Reports	225	500	1 500
Total	20 470	45 000	100 000

5 ESTIMATE OF THE COST OF WORK FOR THE PERIOD APRIL 1992 - JUNE 1992

It is estimated that the cost of the work to be carried out in the next period (until the end of June 1992) will be as budgeted by the NRA.

	£
Staff salaries	13 200
Travel & subsistence	225
Consumables (+ minor capital items)	6 820
Reports	225
Total	20 470

6 PROGRAMME OF WORK FOR THE PERIOD APRIL 1992 - JULY 1992

During the next period until the end of July 1992, the project is expected to proceed to plan *i.e.* Stage 1 will be completed and work will commence on Stage 2.

Monoclonal cell lines will be isolated that secrete antibody to microcystin-LR. Evaluation of these cell lines and the antibodies they produce will lead to the selection of up to five cell lines for antibody production and antibody coupling to a support matrix. Full details are given in the original Biocode Project Proposal (contained in the NRA Research Contract Document).

7 REVIEW OF FACTORS LIKELY TO AFFECT COMPLETION OF THE WORK PROGRAMME

The project is proceeding to plan and there are no known factors at present which are likely to affect the successful conclusion of the project on schedule.

Interim Progress Report **R&D Project 348**

Development of a Field Test Kit for Detection of Blue-Green Algal Toxins

Biocode Limited
August 1992
R&D 348/03/A

CONTENTS

SUMMARY

Work associated with Stage 1 of the project- the production and initial isolation of cell lines secreting antibody to microcystin-LR, has been completed. From the seventeen positive cultures arising from the five fusions performed, 11 monoclonal cell lines that appeared to be producing antibody to microcystin-LR were isolated. These cell lines were isolated on the basis of the secretion of antibody detected using ELISA plates activated with poly-L-lysine and subsequently coated with the unconjugated microcystin-LR.

To date, further evaluation of the eleven monoclonal antibodies has been performed (Stage 2 of the project). Of these, four were found to bind nonspecifically to ELISA plates activated with poly-L-lysine and coated with bovine serum albumin. Six of the remaining seven antibodies that exhibit specific binding to microcystin-LR have been assessed by inhibition ELISA in order to determine their affinity for the microcystin-LR. Difficulties have been found in the application of the inhibition ELISA to poly-L-lysine activated plates coated with the unconjugated microcystin-LR. Using ELISA plates coated with the microcystin-LR-EDA-BSA conjugate, an IC50 of 54ng/ml was determined for the antibody A005-1D 13C6.

In addition to the antibody evaluation studies, the cell lines are currently being expanded in preparation for fermentation. Evaluations of the stability of the hybridoma cells in culture and their monoclonality are ongoing.

The suitability of the immunoaffinity column format for the analysis of samples containing toluene, used in the extraction of microcystin-LR was assessed. Application of up to 0.5%v/v toluene in phosphate buffered saline had no detrimental effect on the use of the Biocode anti-aflatoxin EASI-EXTRACT™ immunoaffinity column.

KEY WORDS

Microcystin-LR, ELISA , Monoclonal Antibody, Affinity.

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.

Biocode Limited is contracted to produce on-site testing kits using monoclonal antibodies. These kits will enable the easy, specific and rapid extraction and identification of microcystin-LR and some structurally related analogues. This work requires the development of novel monoclonal antibodies. Stage 1 of the project includes 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. Stage 2 encompasses selection of hybridoma cells that secrete monoclonal antibodies that bind to microcystin-LR. Selection is based on the application of the enzyme linked immuno sorbent assay (ELISA). This assay is based on the specific attachment of antibodies to ELISA plates coated with conjugated or free microcystin-LR. The affinity of this attachment is then assessed by inhibition ELISA.

2 REVIEW OF TECHNICAL PROGRESS FOR APRIL 1992 - JULY 1992

2.1 Production of monoclonal antibodies to two microcystin-LR conjugates (Stage 1.2)

2.1.1 Isolation of monoclonal cell lines by cloning by limiting dilution.

Fusion cultures identified as producing antibody (detected by ELISA using poly-L-lysine activated plates coated with unconjugated microcystin-LR) were cloned by limiting dilution. The cells in each culture were resuspended and counted using a haemocytometer. The viability of the cells was determined by the exclusion of a vital dye (Trypan Blue). Each cell suspension was diluted to sixty and six viable cells per ml of culture medium, then dispensed across 96-well tissue culture plates containing a layer of splenocyte feeder cells. The plates were incubated at 37°C, 5%v/v CO_2 in air in a humidified incubator. When visible clones of cells appeared, samples of media were taken and assessed by ELISA using poly-L-lysine activated plates coated with unconjugated microcystin-LR. Where possible, culture wells containing single colonies of cells, shown to be producing detectable antibody, were selected for expansion.

2.2 Selection of a range of monoclonal antibodies (Stage 2.1)

2.2.1 Assessment of antibodies for nonspecific binding.

The activation of ELISA plates by poly-L-lysine is a method that has not been used previously at Biocode. The possibility that plate activation could increase nonspecific binding of antibodies was therefore examined.

ELISA plates were prepared as follows:-

i Activation with poly-L-lysine and application of microcystin-LR.

ii Activation with poly-L-lysine and application of bovine serum albumin (BSA).

iii Activation with poly-L-lysine but no application of antigen.

Similarly, plates activated with glutaraldehyde (as indicated in the report R&D 348/02/A) were prepared for comparison. All plates were then incubated for approximately 30 minutes in the presence of 3%w/v BSA in phosphate buffered saline (PBS), pH7.4.

Samples of antibody taken from the cell line cultures were applied undiluted to each of the three ELISA plates and the ELISA was completed. Absorbance values obtained using each of the ELISA plates were compared.

2.2.2 Application of monoclonal antibodies to ELISA plates coated with the microcystin-LR-EDA-BSA plate coating conjugate.

In order to examine the ability of the monoclonal antibodies to detect conjugated microcystin-LR, samples from the cell line cultures were applied to ELISA plates coated with the

microcystin-LR-EDA-BSA conjugate. The samples were applied in serial dilution (ie titrated) and the maximum dilution that indicated a positive result in the ELISA (resulting in an absorbance at 450nm or A_{450} above 0.2) was recorded. The mean A_{450} reading from the maximum dilution was then multiplied by the dilution factor to give a value in "ELISA" units The results were compared to those obtained using poly-L-lysine activated ELISA plates coated with the unconjugated microcystin-LR.

2.2.3 Assessment of antibody affinity by inhibition ELISA.

For the inhibition ELISA a suboptimal concentration of the antibody being assessed (determined by titration ELISA) is applied to the ELISA in the presence of different concentrations of the antigen in soluble form. Microcystin-LR was solubilised first in methanol to a concentration of 10mg/ml, then diluted in PBS, pH 7.4 to a series of concentrations ranging from 5ug/ml to 8ng/ml. Equal volumes of the microcystin-LR solution and diluted antibody were applied simultaneously to the antigen coated ELISA plate. ELISA plates coated with either poly-L-lysine and unconjugated microcystin-LR or the microcystin-LR-EDA-BSA conjugate were used. The ELISA was completed and the inhibitory effect of the addition of soluble antigen to the binding of the antibody to the antigen coated plates was assessed. The concentration of soluble antigen required to reduce antibody binding in the ELISA by 50% is calculated. This is referred to as the IC50 and the lower this value, the higher the affinity of the antibody for the soluble antigen.

2.2.4 Assessment of antibody affinity by application to ELISA plates coated with low concentrations of microcystin-LR.

An alternative means of comparing the affinity of antibodies for microcystin-LR was established. Here, poly-L-lysine activated ELISA plates were coated with decreasing concentrations of microcystin-LR. Plates were coated with microcystin-LR at concentrations ranging from 2ng per ELISA well to 100ng per ELISA well. Only antibodies that had been demonstrated not to bind nonspecifically to poly-L-lysine activated ELISA plates were assessed in this manner.

2.3 Investigation into the effect of toluene on the percentage recovery of aflatoxin immunoaffinity columns

This work was conducted in order to assess the effect that the possible carry over of toluene (from the cell lysis stage) may have on the binding of antigen to immobilised antibody. Samples were prepared in duplicate by addition of 3.5ng of each of the four aflatoxins, AFB_1, B_2, G_1, G_2 to 175ml of PBS pH 7.4 containing 0, 0.05, 0.1, 0.25 and 0.5%v/v toluene. The immunoaffinity columns were prewashed with 20 ml PBS pH 7.4 at 5ml/minute and the samples applied at the same flow rate. Subsequently the columns were washed with 20ml purified water and dried. The bound aflatoxin was eluted with 1.5 ml of methanol and an aliquot from each eluant analysed with standards on a HPLC system. The percentage recovery of the four aflatoxins was calculated.

3 INTERIM RESULTS, DISCUSSION AND CONCLUSIONS

3.1 Production of monoclonal antibodies to two conjugates (Stage 1.2)

3.1.1 Isolation of monoclonal cell lines by cloning by limiting dilution.

The monoclonal cell lines and the fusion from which they were derived are listed in Table 3.1. These cell lines have been identified on the basis of the secretion of antibody detectable on poly-L-lysine activated ELISA plates coated with microcystin-LR. Eleven cell lines in all were isolated successfully.

FUSION	CLONE
A0005-1A	15C4
	17B3
A005-1C/E	9D5
	18C1
	35B2
A005-1D	2D3
	3D1
	4B5
	6D3
	13C6
A005-1F	8D2

TABLE 3.1 Monoclonal cell lines selected for expansion

3.2 Selection of a range of monoclonal antibodies (Stage 2.1)

3.2.1 Assessment of antibodies for nonspecific binding.

Ten of the eleven antibodies listed in Table 3.1 have been assessed for their ability to bind to activated ELISA plates in the absence of the microcystin-LR antigen. Four of the eleven antibodies exhibited nonspecific binding and these are listed in Table 3.2. Nonspecific binding was found to be enhanced on ELISA plates activated with glutaraldehyde. Each of these four antibodies were also applied to ELISA plates coated with the microcystin-LR-EDA-BSA conjugate. Only the A005-1C/E 35B2 antibody gave a positive result (results not shown).

FUSION	CLONE
A005-1A	17B3
A005-1C/E	18C1
A005-1C/E	35B2
A005-1D	2D3

TABLE 3.2 Monoclonal antibodies found to bind non-specifically to activated ELISA plates not coated with microcystin-LR

3.2.2 Application of monoclonal antibodies to ELISA plates coated with the microcystin-LR-EDA-BSA plate coating conjugate.

Six of the seven antibodies shown to bind antigen dependently to poly-L-lysine activated ELISA plates were applied to ELISA plates coated with the microcystin-LR-EDA-BSA conjugate that was originally prepared for use in the ELISA. Samples of tissue culture supernatant were tested as the cultures became sufficiently large to sample. The absorbance reading of a suboptimal dilution of the sample in the ELISA was compared to that obtained using the poly-L-lysine activated ELISA plates coated with unconjugated microcystin-LR. It was the intention that where possible, the conjugate could be used for ELISAs in place of the microcystin-LR, in order to minimise the use of the latter. The results are summarised in Table 3.3.

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

The magnitude of the values presented in Table 3.3 is largely dependent on the concentration of antibody in the sample tested. This is not necessarily a reflection of the suitability of the cell line for antibody production, as it will depend on the confluency of the culture when sampled. The comparative difference of the results obtained when a single sample is applied to the different ELISA plates in the same assay, however, is of greater significance.

3.2.3 Assessment of antibody affinity by inhibition ELISA.

The affinities of each of the antibodies listed in Table 3.3 were assessed by inhibition ELISA. Each antibody was assessed using the appropriate antigen coated ELISA plate and where possible was tested on both the conjugate and microcystin-LR coated plates. The results are presented in Table 3.4.

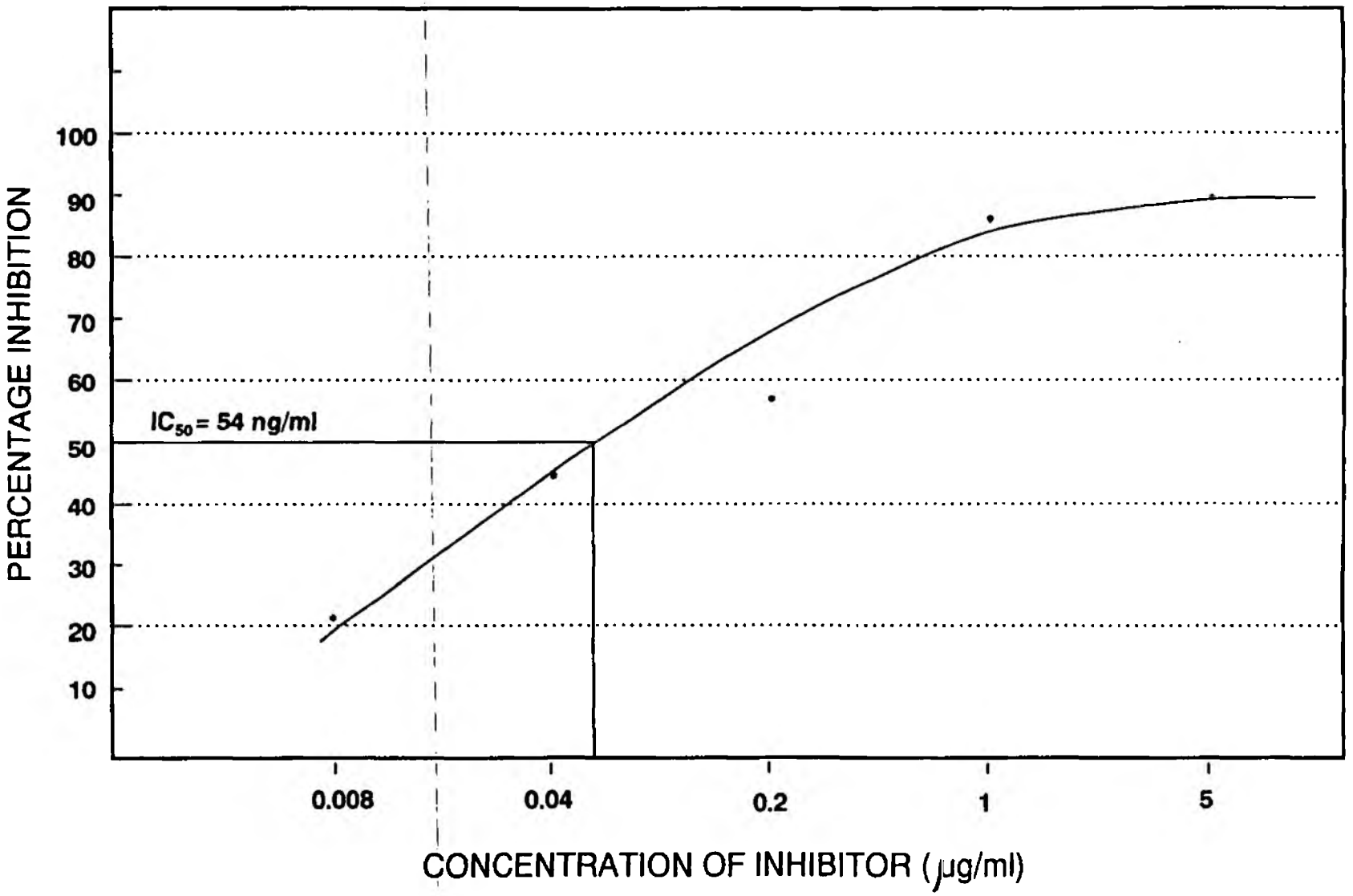
FUSION	CLONE	*IC_{50} (ng/ml)	
		Conjugate coated ELISA plate	Poly-L-lysine activated microcystin-LR coated ELISA plate
A005-1A	15C4	N/A	+0.75
A005-1C/E	9D5	No inhibition	N/A
A005-1D	3D1	No inhibition	No inhibition
A005-1D	4B5	No inhibition	No inhibition
A005-1D	13C6	54	No inhibition
A005-1F	8D2	No inhibition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

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

TABLE 3.4 Assessment of the affinity of antibodies by inhibition ELISA.

Only two antibody affinity values were obtained, one on each type of antigen coated ELISA plate, and one of these values was difficult to reproduce. The inhibition curve for antibody A005-1D 13C6 is shown in Figure 3.1. Although no IC50 value was obtained, each of the other four antibodies were shown (in Section 3.2.1) to bind to the ELISA plate in the presence of the microcystin-LR antigen. It was noted that in some instances, the presence of the solubilised microcystin-LR in the inhibition ELISA actually caused an increase in the binding of the antibody to the ELISA plate rather than a decrease. This effect was observed in several instances when poly-L-lysine activated ELISA plates were used. Furthermore, the antibody A005-1D 3D1 was assessed on microcystin-LR coated ELISA plates that had not been activated with poly-L-lysine, prepared as described in the R&D 348/02/A report, and on conjugate coated ELISA plates. In both instances increased antibody binding was observed in the presence of the microcystin-LR "inhibitor" (results not shown).

FIG 3.1 INHIBITION CURVE FOR A005-1D 13C6



3.2.4 Assessment of antibody affinity by application to ELISA plates coated with low concentrations of microcystin-LR.

Difficulties with the inhibition ELISA (Section 3.2.3) led to an attempt to develop an alternative means of assessing antibody affinity. Here, antibodies A005-1D 3D1 and A005-1D 8D2 were applied to poly-L-lysine activated ELISA plates coated with concentrations of microcystin-LR ranging from 2ng per ELISA to 100ng per ELISA well. Both antibodies were able to detect as little as 2ng of microcystin-LR applied to the ELISA plate and in each instance the absorbance value obtained at a coating level of 100ng per well was not significantly different to that obtained at 2ng per well. No other antibodies have been tested as yet.

3.2.5 Investigation into the effect of toluene on the percentage recovery of aflatoxin immunoaffinity columns

The recovery of aflatoxin immunoaffinity columns in levels of toluene ranging from 0.05% to 0.5%v/v are listed in Table 3.5. Recoveries of all four aflatoxins were greater than 90% in concentrations of toluene up to 0.5%v/v. The results indicate that toluene at these concentrations does not have a measurable effect on the recovery of aflatoxin. It is important to appreciate that other antibody-antigen pairs may behave differently under similar conditions.

% v/v toluene in PBS pH 7.4	AFB_1	AFB_2	AFG_1	AFG_2
0	104.6	99.7	99.7	100.8
0.05	120.1	101.2	103.1	100.9
0.1	114.3	99.5	104.0	98.0
0.25	93.8	95.5	101.0	99.3
0.5	96.9	92.1	99.3	98.5

TABLE 3.5 Evaluation of the sensitivity of aflatoxin EASI-EXTRACT™ columns to aqueous toluene solutions

3.3 DISCUSSION

From the seventeen positives initially detected in the fusion cultures, eleven monoclonal cell lines were isolated that continued to produce antibody that was detectable by poly-L-lysine activated microcystin-LR coated ELISA plates. The loss of cell lines at this early stage, either due to the death of the hybridoma or a loss in antibody production is a common phenomenon often caused by the genetic instability of the hybrid cell.

The monoclonal antibodies were selected by their binding to poly-L-lysine activated ELISA plates coated with unconjugated microcystin-LR. These plates were used in preference to conjugate coated plates as it was argued in the R&D 348/01/A report that these were more selective in the detection of antibodies with an affinity for microcystin-LR.

Four of the eleven cell lines have been rejected on the basis that they produce antibody that binds non-specifically to the poly-L-lysine activated ELISA plates that were used for their selection. However, the problem of nonspecific binding was not unique to the ELISA that utilises the poly-L-lysine activated plates, since the A005-1C/E 35B2 antibody also gave colour on the conjugate coated ELISA plate.

Of the seven antibodies that exhibit antigen dependent binding to poly-L-lysine activated ELISA plates, six have been assessed for their ability to bind to ELISA plates coated with the microcystin-LR-EDA-BSA conjugate. The seventh will be tested when the culture has reached sufficient volume for sampling. Four of the six antibodies tested showed greater binding when applied to the poly-L-lysine activated plates coated with the unconjugated microcystin-LR than with plates coated with the microcystin-LR-EDA-BSA conjugate. It is possible that in most cases the two ELISAs detect distinct antibodies, which is presumably due to the different way in which the microcystin-LR antigen is presented.

One antibody (A005-1D 13C6) has been shown to have an IC50 of 54ng/ml by inhibition ELISA. The difficulties in obtaining affinity data for six other antibodies may not necessarily be because they have poor affinities for the microcystin-LR antigen. One of these antibodies (A005-1A 15C4) was shown on one occasion to have a very high affinity for the antigen (IC50 of 0.75ng/ml), but this was not reproduced. Interestingly, the IC50 value obtained for the A005-1D 13C6 antibody was not obtainable on the poly-L-lysine activated ELISA plates coated with microcystin-LR.

In several instances, the use of microcystin-LR as an inhibitor increased antibody binding in the ELISA. It is possible that microcystin-LR that was applied to the ELISA as an inhibitor is adhering to the ELISA plate, despite the application of BSA to prevent such an interaction, thus causing an increase in antibody binding. This may, in part be due to the microcystin-LR coming out of solution from the PBS used in the ELISA.

The use of ELISA plates coated with low levels of antigen as a measure of antibody affinity requires further evaluation. It is essential that the possibility of non specific binding of the antibody to the poly-L-lysine activated plates is eliminated. In order to interpret how high the affinity of these antibodies is, a direct comparison is required with the A005-1D 13C6 antibody, the IC50 of which has already been determined. This should be straightforward, as this antibody has been shown to bind well to poly-L-lysine activated ELISA plates coated with microcystin-LR. Alternatively, comparisons could be made of the ability of each of the

antibodies to detect low levels of microcystin-LR coated on ELISA plates, and the best ones selected for the next phase of the project.

Studies using aflatoxin immunoaffinity columns indicate that toluene up to a concentration of 0.5%v/v does not have an appreciable effect on antigen-antibody binding.

3.4 CONCLUSIONS

A single monoclonal antibody has been identified as having an IC50 in the region of 50ng/ml. However, a further seven antibodies have been shown to have an affinity for microcystin-LR when coated on poly-L-lysine activated plates. The strength of this affinity remains to be established.

3.5 RECOMMENDATIONS

Assessment of the affinity of antibodies by inhibition ELISA is to be abandoned. Antibody A005-1D 13C6 is to be selected for the next phase of the project. Additional cell lines will be selected on the basis of their ability to detect low concentrations (2ng/ELISA well or less) of microcystin-LR coated on poly-L-lysine activated ELISA plates.

The ultimate assessment of the affinity of the antibodies will be their performance in the immunoaffinity format. As a consequence of the problems with the inhibition ELISA the samples of different variants of microcystin provided by the University of Dundee have not yet been assessed. As these samples are in limited supply it is recommended that they be assessed at a later stage after the antibodies have been coupled to the affinity support.

4 COST OF THE WORK CARRIED OUT IN THE PERIOD APRIL 1992 - JULY 1992

During the third three month period of the project (6 April 1992 - 6 July 1992), the total cost of work carried out is given below and related to the 1992/1993 and total project budgets.

	This Period (£)	1992/1993 Budget (£)	Running Total (£)	Project Total Budget (£)
Staff salaries	13 200	34 000	42 200	67 000
Travel & subsistence	225	500	725	1 500
Consumables (+ minor capital items)	6 820	15 000	21 820	30 000
Reports	225	500	725	1 500
Total	20 470	50 000	65 470	100 000

5 ESTIMATE OF THE COST OF WORK FOR THE PERIOD JULY 1992 - SEPTEMBER 1992

It is estimated that the cost of the work to be carried out in the next period (until the end of September 1992) will be as budgeted by the NRA.

	£
Staff salaries	13 200
Travel & subsistence	225
Consumables (+ minor capital items)	6 820
Reports	225
Total	20 470

6 PROGRAMME OF WORK FOR THE PERIOD JULY 1992 - OCTOBER 1992

Stage 2 of the project will be completed and work will commence on Stage 3.

Efforts will be concentrated on ensuring the monoclonality of selected cell lines prior to their inoculation into the fermenter. Sufficient antibody will be prepared in the fermenter for gel coupling and preliminary evaluation of the immunoaffinity columns produced. As a result of these evaluations a single antibody will be selected for the final stages of the project.

7 REVIEW OF FACTORS LIKELY TO AFFECT COMPLETION OF THE WORK PROGRAMME

Difficulties with the establishment of affinity data for the antibodies has led to an increase in the work requirements. Consequently the work has fallen slightly behind schedule. However, it is likely that the work will be back on course shortly as the cell lines, will soon be ready for inoculation into the fermenter. There are no known factors at present which are likely to affect the successful conclusion of the project and the work is still likely to be completed on schedule.

Interim Progress Report **R&D Project 348**

Development of a Field Test Kit for Detection of Blue-Green Algal Toxins

Biocode Limited
November 1992
R&D 348/04/A

CONTENTS

SUMMARY

This quarter's research has led to the successful isolation of four anti-microcystin-LR monoclonal antibody producing cell lines (Stage 2 of original proposal - Production and coupling of selected monoclonal antibodies to an inert matrix). The work performed over this quarter has concentrated on further evaluation and characterisation of the four selected antibodies and the preparation of hybridoma cells for large scale culture and antibody production.

In addition, further work has focused on assessing the specificity of these antibodies for microcystin-LR. Comparative studies, based on the non-competitive ELISA, involved the application of the antibodies to ELISA plates coated with different variants of microcystin. These tests showed that each of the antibodies bound to at least two of the variants tested. Two antibodies appear to have substantially higher affinities for microcystin 3-desmethyl-RR and microcystin "peak 3" than for microcystin-LR or nodularin. The other two antibodies appeared to have similar affinities for all four variants tested.

Subisotype tests have been performed on the four selected antibodies and each was found to be of the IgM class. Trial purification work, based on precipitation by ammonium sulphate, has led to the establishment of a suitable method for purification of these IgM antibodies.

The four hybridoma cell lines producing these antibodies have been analysed for *mycoplasma* contamination and were found not to be contaminated. Stable, monoclonal hybridoma cell lines have been established for three of these cell lines and the fourth is currently being assessed. The three monoclonal cell lines have been used to establish formal master cell banks in liquid nitrogen, for the provision of cell cultures in the future.

The four key cell lines have been expanded for fermentation and sufficient antibody has now been produced for initial coupling to inert matrices (Stage 2.2).

KEY WORDS

Microcystin-LR, ELISA , Monoclonal Antibody, Affinity.

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 1000 Da. Other structural variants exist that differ by two amino acids.

Biocode Limited is contracted to produce on-site testing kits using monoclonal antibodies. These kits will enable the easy, specific and rapid extraction and identification of microcystin-LR and some structurally related analogues. This work requires the development of novel monoclonal antibodies. Stage 1 of the project includes 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. Stage 2 encompasses selection of hybridoma cells that secrete monoclonal antibodies that bind to microcystin-LR. Selection is based on the application of the enzyme linked immuno sorbent assay (ELISA). This assay is based on the specific attachment of antibodies to ELISA plates coated with free microcystin-LR. Once cell lines have been established as being monoclonal, cultures are expanded for inoculation into a fermenter for *in vitro* antibody production. Monoclonal cultures are also used to establish formal stocks of the cell lines in liquid nitrogen (master cell banks or MCBs) for storage purposes.

2 REVIEW OF TECHNICAL PROGRESS FOR JULY 1992 - OCTOBER 1993

2.1 Expansion of up to five monoclonal antibody producing hybridoma cell lines (Section 2.1)

2.1.1 Expansion of cell lines and assessment of monoclonality.

Monoclonal cell lines that were isolated in the previous report were gradually expanded through a series of culture vessels. Initially, the cells were cultured in the presence of splenocyte feeder cells. The cells were then weaned off the feeder layer and expanded into larger culture vessels.

The monoclonality of the cells was assessed by cloning by limiting dilution. The concentration of cells in a sample of the cell suspension was determined using a haemocytometer and the viability of the cells was determined by the exclusion of a vital dye (Trypan Blue). Each cell suspension was diluted to six cells per ml of culture medium and dispensed across 96-well tissue culture plates containing a layer of splenocyte feeder cells. The plates were then incubated for approximately two weeks at 37°C, 5%v/v CO_2 in air in a humidified incubator. Once colonies were visible, the plates were examined microscopically and a record made of the positions of the clones in the wells. Samples of media were then taken from each well and the presence of antibody determined by ELISA. The percentage correlation of clonal growth and the presence of antibody was calculated. If this did not exceed 95%, the culture was recloned.

Once a culture had been shown to be monoclonal, stocks of the cell line were prepared for storage in liquid nitrogen.

2.1.2 Testing cultures for contamination with *mycoplasma*.

A *mycoplasma* test kit (Boehringer-Mannheim, Lewes, UK) was used to determine whether the cultures of hybridoma cells were contaminated with *mycoplasma*. Samples of culture media were applied directly to the assay which is ELISA based. The assay is able to detect the following:

Mycoplasma arginini
Mycoplasma hyorhinis
Mycoplasma laidlawii
Mycoplasma orale

2.1.3 Preparation of Master Cell Banks (MCB) in liquid nitrogen.

Subconfluent flask cultures (approximately 200 ml) of the monoclonal cell lines were used to prepare the MCB. A bank of at least six vials was prepared for frozen storage, each containing at least 5 X 10^6 viable cells.

2.2 Detection of antibodies by ELISA

The detection of antibody by the antigen specific ELISA was performed using poly-l-lysine

activated PVC ELISA plates, coated at 100 ng per well with the unconjugated microcystin-LR. In order to assess the level of nonspecific binding of selected monoclonal antibodies to these ELISA plates, antibody samples were also applied to poly-l-lysine activated ELISA plates coated with bovine serum albumin (BSA). Monoclonal antibody that bound to the ELISA plate was detected by the application of a second antibody specific to mouse immunoglobulin conjugated to horse radish peroxidase.

2.3 Determination of antibody subclass using the Calbiochem subisotype test kit

Samples taken from selected hybridoma cultures were assayed by using a subisotype test kit (Calbiochem, Nottingham). This kit is ELISA based and is able to identify the following subisotypes of mouse antibody; IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA.

2.4 Antibody production and purification

2.4.1 Preparation of an inoculum for *in vitro* antibody production.

Selected monoclonal cultures were expanded to provide an inoculum for large scale culture. A culture of approximately 200 ml in volume was used to inoculate a Techne stirred vessel or a roller culture for the preparation of sufficient antibody for preliminary gel coupling experiments. Supernatant was harvested from the Techne cultures periodically, and the culture maintained by the addition of fresh culture medium.

2.4.2 Purification of antibody by ammonium sulphate precipitation.

The harvested supernatant was clarified by filtration through a 0.2 μm filter, then concentrated by ultrafiltration (using a molecular weight cut off of 20 KDa) to approximately 10 to 20 times its original volume. An equal volume of saturated ammonium sulphate solution was added to the chilled concentrate. The precipitate was pelleted by centrifugation, resuspended and dialysed in phosphate buffered saline (PBS, pH 7.4). The purity of the final material was assessed by Q Sepharose fast flow ion-exchange chromatography. The purified antibody was applied in 20 mM triethanolamine (pH 7.7). Non-bound material was removed by washing with further 20 mM triethanolamine (pH 7.7). Loosely bound material was removed by washing with 20 mM triethanolamine (pH 7.7) containing 36% v/v 0.2 M NaCl in 20 mM triethanolamine. Bound material was eluted with 20 mM triethanolamine containing 0.5 M NaCl. The presence of protein in the washes and elutions was monitored using an ultraviolet detector.

3 INTERIM RESULTS, DISCUSSION AND CONCLUSIONS

3.1 Expansion of up to five monoclonal antibody producing hybridoma cell lines (Section 2.1)

3.1.1 Expansion of cell lines and assessment of monoclonality.

The five antibodies selected for expansion are listed in Table 3.1. These antibodies were selected on the basis of results presented in the previous report.

CELL LINE /ANTIBODY	PERCENTAGE MONOCLONALITY
A005-1A 15C4	99 +/- 1.5
A005-1D 13C6	93 +/- 4
A005-1D 3D1	To Be Determined
A005-1F 8D2	93 +/- 7.6
A005-1D 4B5	Not Established

TABLE 3.1 Assessment of the monoclonality of A005-1 cell lines

The monoclonality of the cell lines was assessed by cloning by limiting dilution. Each of the cell lines tested so far have been shown to be monoclonal. The presence of antibody able to bind to microcystin was determined by antigen specific ELISA. This ELISA was not quantitative. With the exception of the A005-1D 4B5 cell line, readily detectable levels of antibody were detected in cultures of the expanded cell lines. Expansion of the A005-1D 4B5 cell line was discontinued. Initial frozen stocks for each of the remaining four cell lines were placed in liquid nitrogen.

3.1.2 Testing cultures for contamination with *mycoplasma*.

Samples of cultures of the AOO5-1A 15C4, A005-1D 13C6, A005-1D 3D1 and A005-1F 8D2 when tested for the presence of *mycoplasma* were found to be negative.

3.1.3 Preparation of master cell banks in liquid nitrogen.

Formal MCBs have been set up using monoclonal cultures of cell lines A005-1A 15C4, A005-1D 13C6 and A005-1F 8D2.

3.2 Assessment of the ability of the antibodies to bind to other variants of microcystin

Three additional variants of microcystin (namely nodularin, microcystin 3-desmethyl RR and microcystin "peak 3") were supplied by the University of Dundee. Poly-l-lysine activated plates were coated with the variants, each at 100 ng/ml using the method described in section 2.2.1. Samples of each of the four antibodies were titrated on ELISA plates coated with the variants and the results compared to those obtained when using microcystin-LR as the plate coating antigen. The results in Table 3.3 are presented in "ELISA units" as described in section 3.2 above.

In each case, the level of binding to BSA alone (ie in the absence of specific antigen) was also determined and the figure obtained for this in each case is presented in brackets.

ANTIBODY	Value in ELISA (ELISA units)			
	Microcystin-LR	Nodularin	Microcystin 3-desmethyl-RR	Microcystin peak 3
A005-1A 15C4	1.0 (0.25)	1.19 (0.22)	30.88 (0.26)	48.63 (1.42)
A005-1D 13C6	1.2 (0.35)	1.18 (0.30)	2.00 (0.24)	6.3 (1.04)
A005-1D 3D1	28.0 (10.7)	32.1 (27.38)	43.37 (34.88)	50.25 (34.75)
A005-1F 8D2	1.38 (1.98)	1.32 (1.10)	28.75 (1.72)	32.38 (1.54)

Table 3.3 Antibodies raised to microcystin-LR and their ability to bind to other variants of microcystin

The magnitude of the figures obtained for each antibody is highly dependent on the concentration of antibody in the sample tested. Therefore, direct comparisons of one antibody to the next does not necessarily reflect the suitability of the antibody for the detection of microcystin-LR. However, the same sample of each antibody was used throughout the tests and comparisons can be drawn between the ability of a single antibody to detect the different variants of microcystin.

With the exception of the A005-1F 8D2 antibody, each antibody gave significantly higher colour development in the presence of the microcystin-LR than with the BSA. The A005-1D 3D1 antibody gave consistently high levels of colour in the presence of BSA alone, although in each test, marginally more colour was found in the presence of each microcystin variant tested. Interestingly, although the A005-1D 8D2 antibody gave a poor result with the microcystin-LR, the antibody bound highly significantly to microcystin-3-desmethyl-RR and the microcystin peak 3. The A005-1A 15C4 antibody also gave the highest colour development in the presence of these two variants. The A005-1D 13C6 antibody was able to

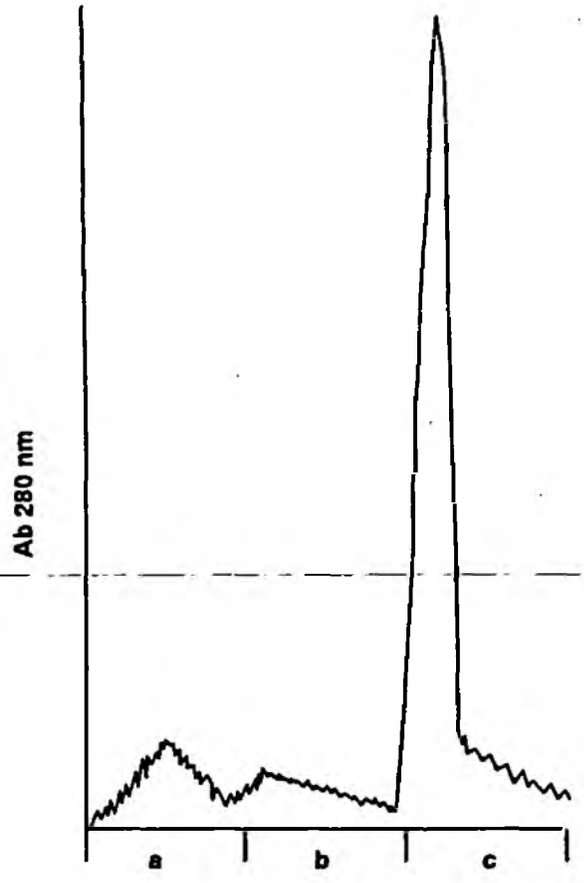
bind significantly to each of the microcystin variants tested.

3.3 Determination of antibody subclass using a subisotype test kit

Samples of cultures of the AOO5-1A 15C4, A005-1D 13C6, A005-1D 3D1 and A005-1F 8D2 were analysed to determine antibody subclass. Each of the four antibodies were found to be of the IgM subclass. In view of this, all further ELISAs were performed using a second antibody that was specific for mouse IgM antibody. It was found that this second antibody gave improved colour development over the original second antibody which recognises all mouse Ig subclasses (results not shown).

3.4 Purification of antibody by ammonium sulphate precipitation

The A005-1D 13C6 antibody was purified by ammonium sulphate precipitation as described in section 2.4.2. The purified sample was then applied to Q Sepharose fast-flow ion-exchange chromatography in order to assess its purity. The chromatograph is presented in Figure 3.1.



Key

a = Non bound material
b = Loosely bound material
c = Antibody peak

Figure 3.1 Analysis of ammonium sulphate purified A005-1D 13C6 antibody by ion exchange chromatography

No significant quantities of protein were found in the non-bound or loosely bound fraction. The peak that eluted with 0.5 M NaCl in 20 mM triethanolamine was applied to the antigen specific ELISA and capture ELISA. This showed that this peak contained the antibody material that actively bound antigen.

3.5 *In vitro* antibody production

Samples of culture supernatant harvested from Techne stirred vessels or roller cultures have been accumulated and the total volumes obtained so far are listed in Table 3.4.

CELL LINE	Total volume of Supernatant (l)
A005-1A 15C4	6.5
A005-1D 13C6	12.9
A005-1D 3D1	12.9
A005-1F 8D2	17.6

Table 3.4 Quantities of culture supernatant collected so far from *in vitro* antibody production

The concentration of antibody in each of the supernatants have yet to be quantified but volumes of this size would normally be sufficient for initial gel coupling studies.

3.6 DISCUSSION

From the results presented in the last report, five hybridoma cell lines were selected for further evaluation on the basis that the antibodies they produced bound to ELISA plates coated with unconjugated microcystin-LR. Each of these antibodies gave significantly greater colour development on ELISA plates coated with specific antigen. Three of these cell lines have now been shown to be monoclonal and stable cell lines have been established. These have been used to set up formal frozen stocks (or MCBs) in liquid nitrogen. A fourth is currently undergoing monoclonality checks. Unfortunately one cell line, A005-1D 4B5, was found to be unstable and antibody production ceased.

Each of the four key antibodies have been found to be of the IgM subclass. Production of an IgM antibody is often associated with a primary immune response of low affinity antibodies. However, in this case, each of the fusions were performed after at least four immunisations and the IC50 of the A005-1D 13C6 antibody was shown in the last report to have a high affinity for the antigen microcystin-LR. The IgM molecule has ten antigen binding sites as opposed to the two found on an IgG molecule. The large number of binding sites may be the reason why it was difficult to establish antigen binding affinities by inhibition ELISA, for some of the antibodies, as stated in the previous report. Conversely, the large number of binding sites could be advantageous in the immunoaffinity format that is to be used in this project.

The results in Table 3.3 illustrate the ability of the four key antibodies to bind to different variants of microcystin. For each antibody being tested, a single sample was used to assess the colour development in the presence of each of the variants. Therefore, the level of colour development of an antibody can be used as a measure of the relative affinity of that antibody for each of the variants and the negative control (BSA). The A005-1D 13C6 antibody appears to have a similar affinity for each of the microcystin variants tested and must therefore recognise the conserved portion of the microcystin molecule, possibly the ADDA sequence. Although the A005-1A 15C4 antibody recognises microcystin-LR, it appears to bind more readily to the microcystin desmethyl-RR and peak three variants. Similarly, the A005-1F 8D2, which was unable to distinguish significantly between microcystin-LR, nodularin and the negative control BSA, appears to recognise the microcystin desmethyl-RR and peak three variants. The peak three variant has recently been shown to be of the LL variety (personal communication with S Bell). The fact that these two antibodies bind differentially between the variants indicates that they bind to a portion of the microcystin that is on or near to the variable region.

Protein A affinity chromatography is a preferred method of purification of antibodies. However, this method is not well suited to the purification of the IgM subclass. Ion-exchange analysis of the A005-1D antibody, isolated by ammonium sulphate precipitation illustrates that the sample is quite pure. The percentage recovery and denaturation of the antibody requires assessment, and this will be performed by a quantitative antigen specific ELISA, once this has been set up.

3.7 CONCLUSIONS

It can be concluded from these results that four monoclonal antibody producing cells have been isolated that recognise microcystin-LR. Two of these antibodies do not differentiate between the four variants of microcystin tested and the other two bind preferentially to two of the variants. Therefore, we have antibodies binding to distinct portions of the microcystin molecule. Stable sources of these antibodies have now been established and three of the cultures have been shown to be monoclonal.

A method of purifying the IgM antibody has been investigated and has been shown to yield pure antibody.

Large quantities of supernatant harvest have been obtained *in vitro*, which can be used for gel coupling and initial evaluation as to the suitability of the antibodies for immunoaffinity kit production.

3.8 RECOMMENDATIONS

Stage 2.2 of the project- antibody coupling- should start immediately, using the antibody material from each of the cell lines, generated in section 3.5 above.

A method of antibody quantification is required which measures the antigen binding activity of the antibodies and can be used to assess the antibody productivity of the cell lines in culture. This will also be used to establish the quantities of antibody required for stage 2.2 of the project.

HPLC analysis of the recovery of microcystin-LR by immunoaffinity columns prepared from each of the antibodies will be the ultimate assessment of the suitability of the four antibodies for the intended kit format. The ability of the antibodies to bind the three alternative variants of microcystin should also be assessed by this method.

4 COST OF THE WORK CARRIED OUT IN THE PERIOD JULY 1992 - OCTOBER 1992

During the third three month period of the project (7 July 1992 - 6 October 1992), the total cost of work carried out is given below and related to the 1992/1993 and total project budgets.

	This Period (£)	1992/1993 Budget (£)	Running Total (£)	Project Total Budget (£)
Staff salaries	8 400	34 000	50 600	67 000
Travel & subsistence	200	500	925	1 500
Consumables (+ minor capital items)	4 200	15 000	26 020	30 000
Reports	700	500	1 425	1 500
Total	13 000	50 000	78 970	100 000

5 ESTIMATE OF THE COST OF WORK FOR THE PERIOD NOVEMBER 1992 - FEBRUARY 1993

It is estimated that the cost of the work to be carried out in the next period (until the beginning of February 1993) will be as budgeted by the NRA.

	£
Staff salaries	8 400
Travel & subsistence	200
Consumables (+ minor capital items)	4 200
Reports	700
Total	13 000

6 PROGRAMME OF WORK FOR THE PERIOD NOVEMBER 1992 - FEBRUARY 1993

Stage 2 of the project will be completed and work will commence on Stage 3.

The following steps will be followed:

1 A quantitative antigen specific ELISA will be established for use to assess the productivity of the cell lines in culture.

2 Antibody already produced *in vitro* will be purified by ammonium sulphate precipitation.

3 The percentage recovery and activity of the antibody purification procedure will be assessed using the quantification ELISA.

4 The four different purified antibodies will be coupled to an immunoaffinity support.

5 Their performance in the immunoaffinity column format, for the detection of the microcystin variants will be established.

6 On the basis of these results, a single antibody will be selected for Stage 3 of the project and large scale antibody production will commence.

7 REVIEW OF FACTORS LIKELY TO AFFECT COMPLETION OF THE WORK PROGRAMME

As each of the four antibodies are of the IgM subclass, the usual method of antibody purification has had to be adapted. Modifications have also been required of the ELISA. These difficulties have been successfully overcome, but have required additional effort. It is possible that adaptations to our normal method of antibody coupling will also have to be investigated.

QUALITY REVIEW INVITATION

PM3/cp

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 (Project Record)

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

Product Code(s)	Product Name(s)
348/7/A	Draft Final Report - Development of a field test kit for detection of blue-green algal toxins

Venue (if postal enter "POSTAL")	Postal	Date Time Duration	19/12/ 94 **closes: 10/1/95**
Chairman	Paul Williams	Project Manager	M Pearson
Reviewers	P Williams (SW) M Bramley (HO) J Seager (HO) T Long (HO) M Pearson (A) G Codd (External advisor, Toxic Algal Group) A Ferguson (A) G Brighty (A)		

Product Description(s) attached

Draft Final Report (Project Record) accompanies 348/6/A summarising the work undertaken on Project 348. It contains the fully detailed technical Interim Reports (5 no) presented during the project and are to be bound as a single volume Project Record. This report 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 only.
The Draft Final Report (Project Record) and final version should remain Externally Restricted due to a confidentiality agreement between NRA and Biocode.

Product(s) attached (where applicable)

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 ?

2 Of the information presented, is it clear to the NRA audience what was undertaken and achieved ?

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 Recommend dissemination of final R&D Note and Project Record

Interim Progress Report R&D Project 348

Development of a Field Test Kit for Detection of Blue-Green Algal Toxins

Biocode Limited
February 1993
R&D 348/05/A

CONTENTS

SUMMARY

During the period November 1992 to January 1993, work has continued on Stage 2 of the original proposal - production and coupling of selected monoclonal antibodies to an inert matrix. In this quarter we have continued to characterise the four antibodies which were selected for their ability to bind to microcystin-LR.

Optimisation of the competitive ELISA led to determination of an IC_{50} of 77 ng/ml for the antibody A005-1D 13C6. However, no IC_{50} was established for the other three key antibodies (A005-1A 15C4, A005-1D 3D1 and A005-1F 8D2). Consequently, efforts have been concentrated on the application of the A005-1D 13C6 antibody to kit formatting.

The A005-1D 13C6 antibody has been coupled to cyanogen bromide and triazine activated supports with the aim of producing immunoaffinity (EASI-EXTRACT™) columns for the detection of microcystin-LR. When solutions of microcystin-LR were applied to the immunoaffinity columns, no antigen binding was found. Alternative coupling procedures are being investigated and different kit formats are to be considered. Furthermore, an alternative procedure for the purification of the IgM antibody has been developed.

KEY WORDS

Microcystin-LR, ELISA , Monoclonal Antibody, Affinity , HPLC, 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 1000 Da. Other structural variants exist that differ by two amino acids.

Biocode Limited is contracted to produce on-site testing kits using monoclonal antibodies. These kits will enable the easy, specific and rapid extraction and identification of microcystin-LR and some structurally related analogues. This work requires the development of novel monoclonal antibodies. Stage 1 of the project includes 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. Stage 2 encompasses selection of hybridoma cells that secrete monoclonal antibodies that bind to microcystin-LR. Selection is based on the application of the enzyme linked immuno sorbent assay (ELISA). This assay is based on the specific attachment of antibodies to ELISA plates coated with free microcystin-LR. Once cell lines have been established as being monoclonal, cultures are expanded for inoculation into a fermenter for *in vitro* antibody production. The latter part of Stage 2 encompasses the purification of the antibody produced *in vitro* and coupling of this antibody to a solid support in a prototype kit format.

2 REVIEW OF TECHNICAL PROGRESS FOR NOVEMBER 1992 - FEBRUARY 1993

2.1 Further evaluation of selected monoclonal antibodies

2.1.1 The quantification ELISA.

The detection of antibody by the antigen specific ELISA was performed using poly-l-lysine activated PVC ELISA plates, coated at 100 ng per well with the unconjugated microcystin-LR. Monoclonal antibody that bound to the ELISA plate was detected by the application of a second antibody specific to mouse IgM conjugated to horse radish peroxidase. In order to establish a means of antibody quantification, standards of known concentration of antibody were prepared. The concentration of a sample of the A005-1D 13C6 antibody that had been purified by ammonium sulphate precipitation was determined by absorbance at 280 nm. This sample was analysed by ELISA as a series of dilutions and appropriate concentrations were selected in order to give a good standard curve. Batches of the appropriate concentrations of purified antibody were then aliquotted and stored frozen. Vials of the standards were retrieved from frozen and tested alongside the same batches of standards that had not been frozen to establish the effect of freezing. The concentration of antibody assigned to the frozen standards was adjusted to compensate for the effects of the freeze-thaw cycle. This quantification ELISA was used to determine the concentration of antibody containing samples by ELISA.

2.1.2 Assessment of antibody affinity by competitive ELISA.

The affinity of the monoclonal antibodies to microcystin-LR was assessed by competitive ELISA, using ELISA plates coated with the microcystin-LR conjugated to bovine serum albumin (BSA). In order to optimise this ELISA, plates were coated with different concentrations of the conjugate (50, 100, 200 and 400 ng/well). Two different second antibodies (conjugated to horseradish peroxidase) were also tested, again at different concentrations. One antibody recognises all mouse immunoglobulins and was tested at dilutions of 1/2500, 1/5000, and 1/10000. The other is specific to mouse IgM antibody and was tested at dilutions of 1/2000, 1/4000, and 1/8000. Samples of each of the four key antibodies were titrated under each of the conditions and the optimum antigen coating level and second antibody dilution was determined. Using the appropriate conditions, a sample of each of the antibodies was applied at a suboptimal dilution to the competitive ELISA in the presence of different concentrations of the antigen microcystin-LR. The concentration of microcystin-LR required to reduce antibody binding by 50% (IC_{50})was determined.

2.1.3 Determination of antibody subclass using the Calbiochem subisotype test kit.

The subisotype of antibody within a sample was determined using an ELISA based test kit (Calbiochem, Nottingham). This kit is able to identify antibodies of the following subisotypes: IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA.

2.2 Expansion of up to five monoclonal antibody producing hybridoma cell lines (Section 2.1)

2.2.1 Maintenance of the cell lines in large scale culture and antibody production.

Techne stirred culture vessels and roller cultures of the monoclonal cell lines that were initiated in the previous quarter were maintained so that they continued to produce antibody required for gel coupling experiments. The cell lines were maintained in culture medium supplemented with a synthetic serum substitute (Nuserum) in order to aid the purification of the antibody.

2.2.2 Antibody purification by ammonium sulphate precipitation.

The harvested supernatant was clarified by filtration through a 0.2 μm filter, then concentrated by ultrafiltration (using a molecular weight cut off of 20 KDa) to approximately 10 to 20 times its original volume. An equal volume of saturated ammonium sulphate solution was added to the chilled concentrate. The precipitate was pelleted by centrifugation, resuspended and dialysed in phosphate buffered saline pH 7.4 (PBS).

2.2.3 Purification of antibody by ion-exchange chromatography.

The antibody to be purified was clarified by filtration through a 0.2 μm filter, then concentrated by ultrafiltration (using a molecular weight cut off of 20 KDa) to approximately 10 to 20 times its original volume. The sample of antibody was applied to a Q Sepharose (Pharmacia, UK) fast flow ion-exchange column in 20 mM triethanolamine (pH 7.7). Non-bound material was removed by washing with further 20 mM triethanolamine (pH 7.7). Loosely bound material was removed by washing with 20 mM triethanolamine (pH 7.7) containing 36% v/v 0.2 M NaCl in 20 mM triethanolamine. Bound material was eluted with 20 mM triethanolamine containing 0.5 M NaCl. The presence of protein in the washes and elutions was monitored using an ultraviolet detector.

2.3 Coupling of antibodies to solid supports and evaluation of microcystin-LR binding ability (section 2.2)

2.3.1 Coupling antibodies to cyanogen bromide-activated Sepharose 4B and incorporation into EASI-EXTRACT™ columns.

The A005-1D 13C6 ammonium sulphate purified antibody was linked to the support matrix at various coupling concentrations. The cyanogen bromide-activated Sepharose 4B (Pharmacia, UK) was allowed to swell for approximately 15 minutes in 1 mM HCl and then further washed with 1 mM HCl and coupling buffer (0.1 M sodium hydrogen carbonate, 0.5 M sodium chloride pH 8.0). The antibody solution diluted in PBS (pH 7.4) was mixed with the Sepharose gel suspended in an equal volume of coupling buffer. The coupling reaction was incubated for 1.5 hours at room temperature on an orbital shaker. The supernatant was then filtered off and the absorbance at 280 nm measured to determine the efficiency of the binding of the antibody to the support matrix. Any remaining active sites on the cyanogen bromide activated Sepharose 4B were subsequently blocked by incubation with 1 M ethanolamine

pH 8.0 for a further 1.5 hours. The blocked gel was washed alternately with coupling buffer and 1 mM HCl until the absorbance (at 280 nm) of the filtrate was zero ± 0.01. The gel was finally washed with PBS containing 0.05% w/v sodium azide and stored at 2-8°C (as a 50% v/v settled gel suspension).

EASI-EXTRACT™ columns were prepared by adding 1 ml of the 50% v/v gel suspension into a column body containing a bottom frit. A second frit was placed on top of the gel and the column finally washed through with PBS containing 0.05% v/v azide.

2.3.2 Coupling antibodies to triazine activated agarose 4XL and incorporation into EASI-EXTRACT™ columns.

The A005-1D 13C6 ammonium sulphate purified antibody was also linked to a triazine activated agarose (Pharmacia) support matrix at various coupling concentrations. The method is essentially that described for cyanogen bromide-activated Sepharose 4B with the following deviations. The triazine activated agarose was swollen in purified water instead of 1 mM HCl and the coupling performed for 2 hours at room temperature in 25 mM acetate buffer pH 5.0. The remaining active sites on the gel were blocked by overnight incubation with 2 M ethanolamine pH 9.5. Finally the gel was washed with purified water and 20% v/v ethanol in purified water and stored in this latter buffer.

2.3.3 Evaluation of the prepared immunoaffinity (EASI-EXTRACT™) columns to bind antigen (microcystin-LR).

An HPLC method was developed for the quantitative analysis of microcystin-LR This method was essential for assessing the immunoaffinity columns for their antigen binding ability. The method uses an ODS2 reverse phase HPLC column (25 cm x 4.6 mm) and a gradient run over 22 minutes from 30% v/v to 50% v/v acetonitrile in HPLC grade water containing 0.05% v/v trifluoroacetic acid (TFA). The eluate from the column was monitored at 238 nm with a UV/visible absorbance detector. The retention time for microcystin-LR was approximately 16 minutes.

The immunoaffinity columns were assessed for their ability to bind and recover microcystin-LR. Binding capacity was measured by overloading the EASI-EXTRACT™ columns with 10 μg of microcystin-LR in 50 ml of PBS or PBS containing 10% v/v methanol. Similarly the recovery was determined when 40 ng of microcystin-LR was applied to a column in 50 ml of PBS or PBS containing 10% v/v methanol.

After the EASI-EXTRACT™ column had been washed with 20 ml PBS, the samples, either capacity or recovery, were applied to the column at a flow rate of 5 ml/minute. The columns were subsequently washed with 20 ml HPLC grade purified water and the bound microcystin-LR eluted with 1.5 ml neat methanol. Aliquots of the methanol eluate were then diluted to 43% v/v with HPLC grade purified water and injected on to the HPLC system. The peak area was determined and compared with the peak area of the standard containing a known quantity of microcystin-LR. From this the quantity of microcystin-LR bound to the column could be calculated.

3 INTERIM RESULTS, DISCUSSION AND CONCLUSIONS

3.1 Further evaluation of selected monoclonal antibodies

3.1.1 The quantification ELISA.

The standard curve for the ELISA is presented in Figure 3.1. The concentration of antibody in these standards is greater than that required to give a standard curve when using an antibody of the IgG subclass. This is explained by the fact that an antibody of the IgM subclass has a relative molecular mass that is more than five fold higher than that of an IgG antibody. This standard curve was used to quantify a variety of antibody samples such as those used for gel coupling.

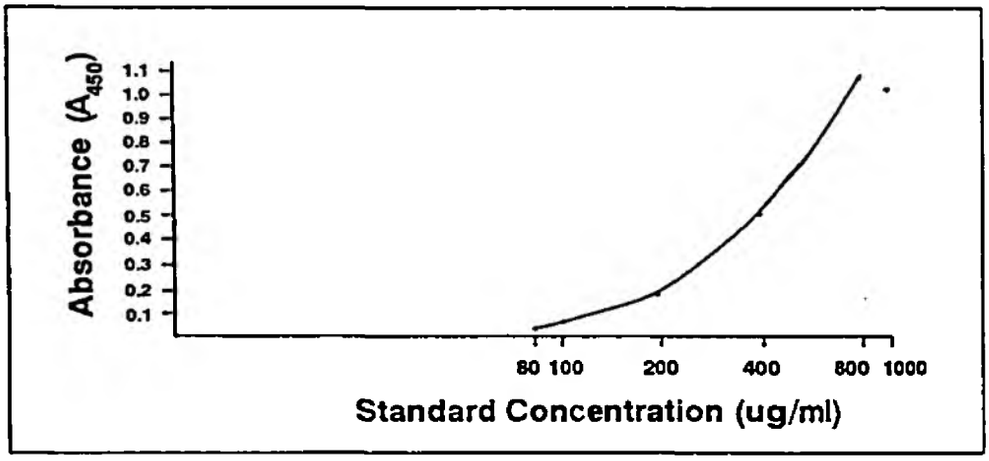


Figure 3.1 Standard curve for the quantification ELISA

3.1.2 Assessment of antibody affinity by competitive ELISA.

In order to enhance the sensitivity of the ELISA which utilises plates coated with microcystin-LR conjugated to BSA, measures were taken to optimise the antigen coating level and the application of second antibody.

The optimum concentration of antigen coating of the ELISA plates was first determined and it was found that coating levels that exceeded 100 ng/well caused nonspecific binding of each of the second antibodies (Table 3.1). Similarly, application of the anti-IgM second antibody at dilutions below 1/4000 led to substantial nonspecific binding to the plate coating conjugate (Table 3.1b). The anti-mouse immunoglobulin second antibody was found to be more specific and gave very little background colour at each of the dilutions tested (Table 3.1a).

Conjugate Coating Level (ng/well)	Second Antibody Dilution		
	1/2500	1/5000	1/10000
50	0.145	0.108	0.129
100	0.193	0.128	0.115
200	0.287	0.215	0.144
400	0.382	0.250	0.161

TABLE 3.1 Level of nonspecific binding of second antibody in the ELISA

a) binding of anti-mouse immunoglobulin second antibody to the ELISA plate in the absence of sample antibody

Conjugate Coating Level (ng/well)	Second Antibody Dilution		
	1/2000	1/4000	1/8000
50	0.423	0.208	0.117
100	0.630	0.300	0.196
200	0.989	0.502	0.266
400	1.162	0.552	0.290

b) binding of anti-mouse IgM second antibody to the ELISA plate in the absence of sample antibody

The optimum conditions for detection of each of the four key antibodies (A005-1A 15C4, A005-1D 3D1, A005-1D 13C6 and A005-1F 8D2) was established by applying serial dilutions of the test antibodies under the conditions described above.

The four key antibodies were applied to the optimised ELISA at suboptimal dilutions in the presence of different concentrations (10, 50, 100 and 1000 ng/ml) of microcystin-LR solubilised in PBS (pH 7.4) containing 10% v/v methanol. An IC50 value of 77 ng/ml was established for the A005-1D 13C6 antibody and the inhibition curve is presented in Figure 3.2. The other three antibodies (A005-1A 15C4, A005-1D 3D1 and A005-1F 8D2) were not inhibited by the microcystin-LR and no IC50 values could be determined.

The sample of A005-1D 13C6 antibody applied to the competitive ELISA was determined, by quantification ELISA to be approximately 0.5 mg/ml. Examination of the inhibition curve suggests that this concentration of antibody is saturated by between 100 ng/ml and 1 μg/ml of the microcystin-LR antigen.

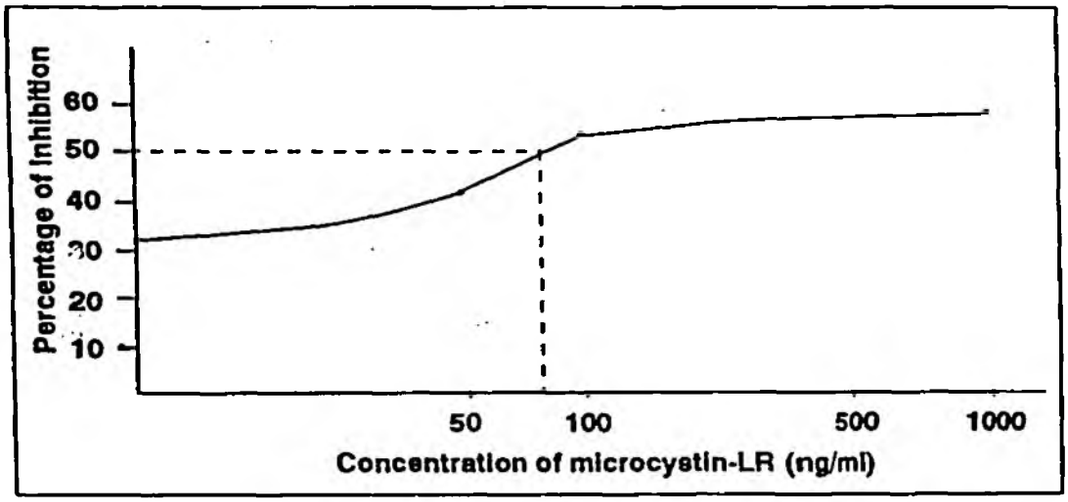


Figure 3.2 Inhibition of the A005-1D 13C6 antibody by microcystin-LR in the competitive ELISA

3.2 Expansion of up to five monoclonal antibody producing hybridoma cell lines (Section 2.1)

3.2.1 Maintenance of the cell lines in large scale culture and antibody production.

Samples of culture supernatant harvested from Techne stirred vessels or roller cultures have been accumulated and the total volumes obtained so far are listed in Table 3.2.

Cell Line	Total volume of Supernatant (l)
A005-1D 13C6	22.6
A005-1A 15C4	11
A005-1D 3D1	12.9
A005-1F 8D2	17.6

TABLE 3.2 Quantities of culture supernatant collected so far from *in vitro* antibody production

Samples of harvest from the culture of the A005-1D 13C6 antibody have been applied to the quantification ELISA and have been shown to contain in the region of 200 μg/ml of active antibody.

In order to monitor the stability of the subclass of the antibody produced by the A005-1D 13C6 cell line during fermentation, a sample of the Techne culture was applied to the subisotype test kit. The sample was found only to contain antibody of the IgM subclass

3.3 Coupling of antibodies to solid supports and evaluation of microcystin-LR binding ability

3.3.1 Coupling antibodies to cyanogen bromide-activated Sepharose 4B and incorporation into EASI-EXTRACT™ columns.

The ammonium sulphate purified antibody A005-1D 13C6 was coupled to cyanogen bromide-activated Sepharose 4B at various concentrations with the aim to optimise the binding capacity of the gel for microcystin-LR. These coupling concentrations were determined both by quantification ELISA and absorbance measurements at 280 nm and the figures correlated closely. The coupling efficiency of the antibodies to the gel matrix was assessed by taking absorbance measurements at 280 nm of the antibody fluid before coupling and the gel filtrate after coupling. The coupling efficiencies of the two antibodies to the gel matrix are detailed in Table 3.3. As expected the coupling efficiency of the antibody to the gel matrix decreases with the coupling concentration of antibody used. This occurs because the cyanogen bromide-activated Sepharose 4B has only a limited number of reactive sites on its surface and the amount of antibody in the coupling reaction soon exceeds the number of such sites available on the support matrix.

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 3.3 The coupling efficiency of the antibodies to cyanogen bromide-activated Sepharose 4B

3.3.2 Evaluation of the cyanogen bromide-activated Sepharose 4B immunoaffinity (EASI-EXTRACT™) columns to bind antigen (microcystin-LR).

The HPLC method developed for the quantitative analysis of microcystin-LR gave a retention time of approximately 16 minutes for the toxin. A typical chromatogram is shown in Figure 3.3. The minimum detection limit using this method was less than 5 ng of microcystin-LR.

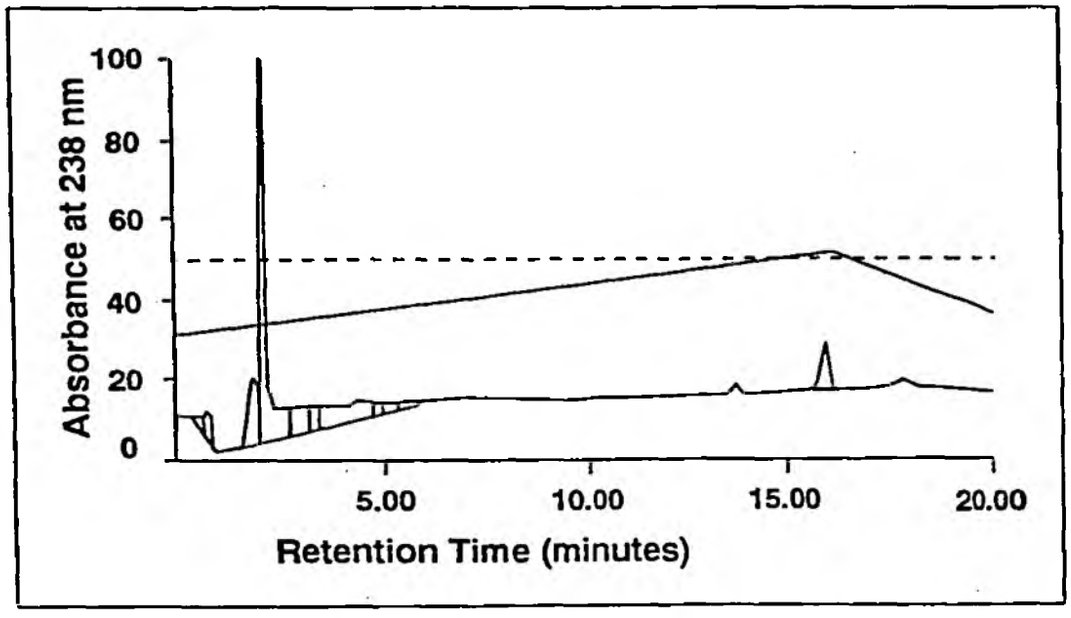


Figure 3.3 HPLC chromatogram of microcystin-LR

Binding capacity tests were performed on the EASI-EXTRACT™ columns prepared from the A005-1D 13C6 immunoaffinity gel. The tests were conducted by applying 10 μg of microcystin-LR in PBS, borate/KCl buffer pH 8.5, 10% v/v and 20% v/v methanol or acetonitrile in PBS. In all cases none of the EASI-EXTRACT™ columns had any measurable binding capacity for microcystin-LR ie no binding of the antigen to the immunoaffinity gel was observed. Samples of the solution prior to and after application to the column were also analysed. The results indicated that all of the microcystin-LR passed straight through the EASI-EXTRACT™ column. As expected when the EASI-EXTRACT™ columns were tested for percentage recovery of microcystin-LR (by applying 40 ng) no binding of the antigen to the gel was observed.

In an attempt to obtain microcystin-LR binding to the immunoaffinity gel an aliquot of the A005-1D 13C6 immunoaffinity gel was incubated with 10 μg of microcystin-LR in PBS for 1 hour at room temperature. After this time the gel was prepared into an EASI-EXTRACT™ format column and the column washed with 20 ml purified water. The column was subsequently dried with air and any bound microcystin-LR eluted with 1.5 ml neat methanol. The eluate contained less than 50 ng of microcystin-LR when analysed by HPLC but when the controls (aflatoxin immunoaffinity gel and blocked cyanogen bromide-activated Sepharose gel with no antibody bound) were examined a comparable quantity of microcystin-LR was found to bind. This indicates that the binding observed was probably non-specific in nature.

3.3.3 Coupling antibodies to triazine activated agarose 4XL and incorporation into EASI-EXTRACT™ columns.

The A005-1D 13C6 antibody has also been coupled to triazine-activated agarose 4XL activated at 5-10 μmol/g and 15-25 μmol/g each at 20 mg of antibody per ml of swollen gel. The coupling efficiency was in both cases about 60% indicating that a large number of the sites on the gel were occupied with antibody.

3.3.4 Evaluation of the triazine-activated agarose immunoaffinity (EASI-EXTRACT™) columns to bind antigen (microcystin-LR).

Further binding capacity tests were conducted (by applying 10 μg of microcystin-LR in PBS and 10% v/v methanol in PBS) on the EASI-EXTRACT™ columns prepared from the A005-1D 13C6 antibody linked to triazine activated agarose. Once again no measurable binding capacity for microcystin-LR was measured in any of the gels.

3.3.5 Investigation into the pH stability of the A005-1D 13C6 antibody.

The A005-1D 13C6 antibody was tested using a quantification ELISA for its pH and ionic strength stability by simulating the conditions during the coupling process. One possibility was that the IgM antibody was pH sensitive especially to acidic conditions such as those used to wash the gel after the coupling and the blocking process ie 1 mM HCl or to the high salt conditions used for blocking ie 1 M ethanolamine pH 8.0. To assess this immunoaffinity gels were prepared with the A005-1D 13C6 antibody and cyanogen bromide-activated Sepharose 4B in which the washing reagent 1 mM HCl was replaced with nanopure water and in which

the blocking step was eliminated totally.

Once again binding capacity tests did not detect any microcystin-LR binding to the immunoaffinity gel.

3.3.6 Comparison of purification of the A005-1D 13C6 antibody by ammonium sulphate precipitation and ion-exchange chromatography.

A single batch of the A005-1D 13C6 antibody that had been harvested from Techne and roller cultures was concentrated by ultrafiltration using membranes with a molecular weight cut off of 20,000 Da. This batch of concentrate was divided. Half was purified by ammonium sulphate precipitation and the other half by ion-exchange chromatography. Samples of the starting material and antibody purified by each method were quantified by ELISA. The percentage recovery of antibody that actively binds antigen was calculated for the two methods of purification (Table 3.4).

Both methods of purification recovered in excess of 100% of active antibody. This is explained by the renaturation of antibody during the purification process. The results presented in Table 3.4 indicate that both methods of purification yield antibody that actively binds antigen.

Method of Purification	Quantity of Antibody* (mg)		Percentage Recovery
	Before Purification	After Purification	
Ammonium Sulphate Precipitation	339	558	165
Ion-exchange Chromatography	319	356	112

* Determined by quantification ELISA

TABLE 3.4 Comparison of the purification of A005-1D 13C6 antibody by ammonium sulphate precipitation and ion-exchange chromatography

3.6 DISCUSSION

Previous reports have indicated that an IC50 value could be determined for the A005-1D 13C6 antibody by competitive ELISA, when ELISA plates coated with microcystin-LR conjugated to BSA were used. Attempts at establishing the affinities of the other three key antibodies had been unsuccessful, due to poor colour development on ELISA plates coated with the conjugate.

As we had since accumulated larger quantities of the antibodies at higher concentrations it was decided that this work should be repeated. The ELISA was optimised for each of the antibodies to be tested. Application of each of the four antibodies to optimised competitive ELISAs led to the reproduction of the initial IC_{50} value for the A005-1D 13C6 antibody. However, no affinity data was obtained for the other three antibodies (A005-1A 15C4, A005-1D 3D1 and A005-1F 8D2). There is no evidence that these three antibodies have a high affinity for the antigen microcystin-LR. The IC_{50} value obtained for the A005-1D 13C6 antibody is comparable to that found for the other antibodies used successfully in the EASI-EXTRACT™ column format. As a consequence it was decided that efforts should be concentrated on the coupling of the A005-1D 13C6 antibody to solid supports.

The quantification ELISA has been used to determine the concentration of antibody in the Techne culture of the A005-1D 13C6 antibody. This concentration of 200 μg/ml compares well with the productivity of other cell lines that have been used as a source of antibody for the manufacture of EASI-EXTRTACT™ columns. Hybridoma cells that secrete antibody of the IgM subclass are reported to undergo switching of the subclass of antibody they produce. Application of a sample from the Techne culture of the A005-1D 13C6 cell line to the subisotype test showed that only antibody of the IgM subclass was present after prolonged culture.

In the previous report, it was shown that purification of the A005-1D 13C6 antibody by ammonium sulphate precipitation yielded antibody that was active and quite pure. Coupling efficiencies of gels prepared at coupling levels below 10 mg/ml of antibody per ml of swollen gel indicate that the antibody was chemically linked to the support matrix. At higher coupling concentrations the efficiency of coupling decreases as a consequence of the limited number of active sites on the support matrix. It appears that 1 ml of gel is saturated with approximately 12 mg of antibody and this figure is comparable to that of IgG couplings performed routinely.

Despite the apparent success of the coupling procedure the immunoaffinity column that was prepared from the coupled gel bound no detectable quantity of the microcystin-LR antigen. Prior to coupling, the A005-1D 13C6 antibody was assessed by quantification ELISA and shown to bind antigen. The pH changes to which the antibody is subjected to during the coupling procedure were examined and found to have no effect on the antigen binding activity of the A005-1D 13C6 antibody. A number of modifications to the coupling procedure were assessed as well as alternatives to the methods of column evaluation, with no success as yet.

By competitive ELISA it has been shown that 1 mg of the A005-1D 13C6 antibody in free solution will bind between 200 ng and 2 μg of microcystin-LR. By calculation of the relative molecular masses of the IgM antibody (1000 KDa) and microcystin-LR (1 KDa), it can be

established that 1 mg of the antibody should bind up to 5 μg of microcystin-LR. This indicates that the antibody used for gel coupling was not fully active. Despite this the 10 mg of antibody contained within each immunoaffinity column should bind detectable levels of microcystin-LR. This suggests that careful handling of the IgM antibody is required and alternative methods of antibody purification and antibody immobilisation should be considered.

The preferred method of antibody purification is protein A affinity chromatography which yields antibody of high purity. Unfortunately antibodies of the IgM subclass are not well suited to this method of purification. Although ammonium sulphate purified A005-1D 13C6 antibody was shown to be quite pure, it would be advantageous to improve the purity of the antibody used for coupling. Here, the ion-exchange method was shown to yield antibody of high antigen binding activity.

3.7 CONCLUSIONS

The cell line A005-1D 13C6 has been shown to produce sufficient quantities of antibody to be considered for immunoaffinity column production. Of the four antibodies isolated, only this antibody has been shown to have a high affinity for microcystin-LR.

The A005-1D 13C6 antibody has been chemically linked to the support matrix and incorporated successfully into immunoaffinity (EASI-EXTRACT™) columns. The EASI-EXTRACT™ columns prepared using the A005-1D 13C6 antibody appear to have no measurable binding capacity for microcystin-LR. Some of the factors which may be responsible for this have been examined, but further factors remain to be investigated.

3.8 RECOMMENDATIONS

The remainder of the work in this project is to be concentrated solely on the formatting of the A005-1D 13C6 antibody.

Alternative methods of antibody purification are to be investigated. Work should continue with the aim of developing a functional immunoaffinity format kit. Alternative membrane based kit formats will be investigated in parallel.

4 COST OF THE WORK CARRIED OUT IN THE PERIOD NOVEMBER 1992 - FEBRUARY 1993

During the third three month period of the project (7 November 1992 - 6 February 1993), the total cost of work carried out is given below and related to the 1992/1993 and total project budgets.

	This period (£)	1992/1993 Budget (£)	Running Total (£)	Project Total Budget (£)
Staff salaries	8,400	34,000	59,000	67,000
Travel and subsistence	200	500	1,125	1,500
Consumables (+ minor capital items)	4,200	15,000	26,220	30,000
Reports	700	500	2,125	1,500
TOTAL	13,000	50,000	78,970	100,000

5 ESTIMATE OF THE COST OF WORK FOR THE PERIOD MARCH 1993 - JUNE 1993

It is estimated that the cost of the work to be carried out in the next period (until the beginning of June 1993) will be as budgeted by the NRA.

	£
Staff salaries	16,400
Travel and subsistence	575
Consumables (+ minor capital items)	3,980
Reports	575
TOTAL	21,530

6 PROGRAMME OF WORK FOR THE PERIOD MARCH 1993 - JUNE 1993

Work will continue on Stage 2 of the project.

The following steps will be followed:

1 The A005-1D 13C6 cell line will be transferred to a hollow fibre fermenter for the production of sufficient antibody for use in kit formatting.

2 The A005-1D 13C6 antibody will be purified by ion-exchange chromatography.

3 The ion-exchange purified antibody will be coupled to immunoaffinity supports and the performance evaluated extensively.

4 In parallel, a membrane based format for the application of the A005-1D 13C6 antibody to the detection of microcystin-LR will also be investigated.

7 REVIEW OF FACTORS LIKELY TO AFFECT COMPLETION OF THE WORK PROGRAMME

As suspected in our previous report we have experienced difficulties in preparing functional immunoaffinity (EASI-EXTRACT™) columns for the detection of microcystin-LR. Additional efforts are therefore required in the next Stages to format suitable kits for the effective use of the selected monoclonal antibody.