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

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

Biocode Limited
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R&D 348/05/A

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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.

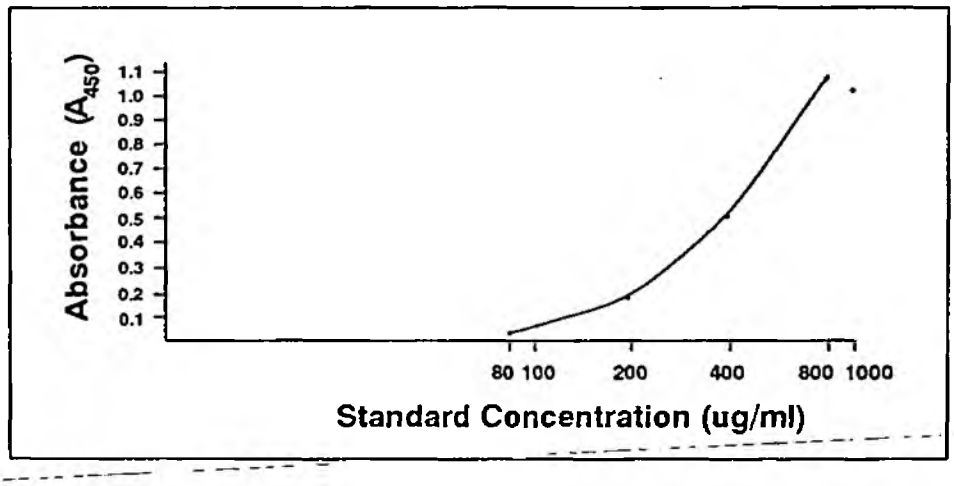

Figure 3.1 Standard curve for the quantification ELISA

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.

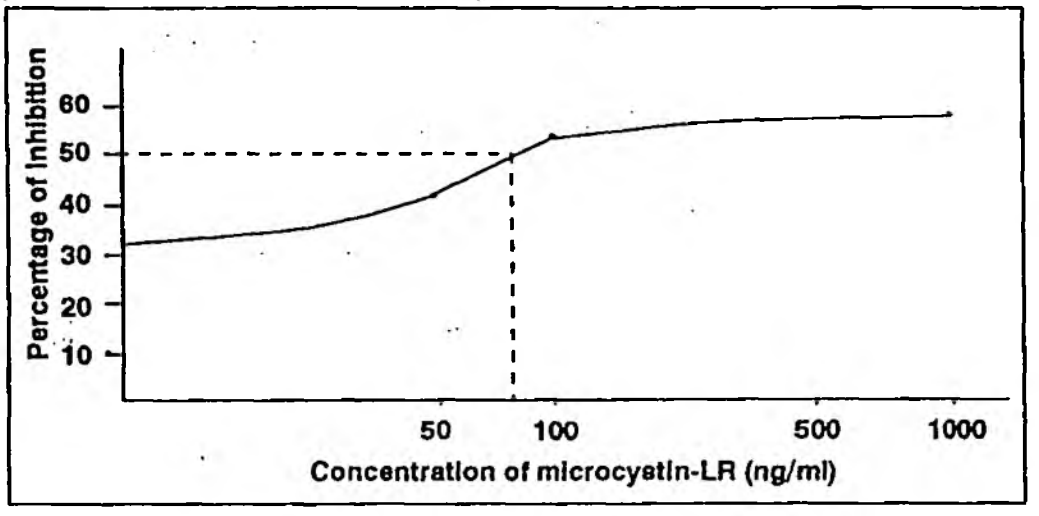

Figure 3.2 Inhibition of the A005-1D 13C6 antibody by microcystin-LR in the competitive ELISA

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.

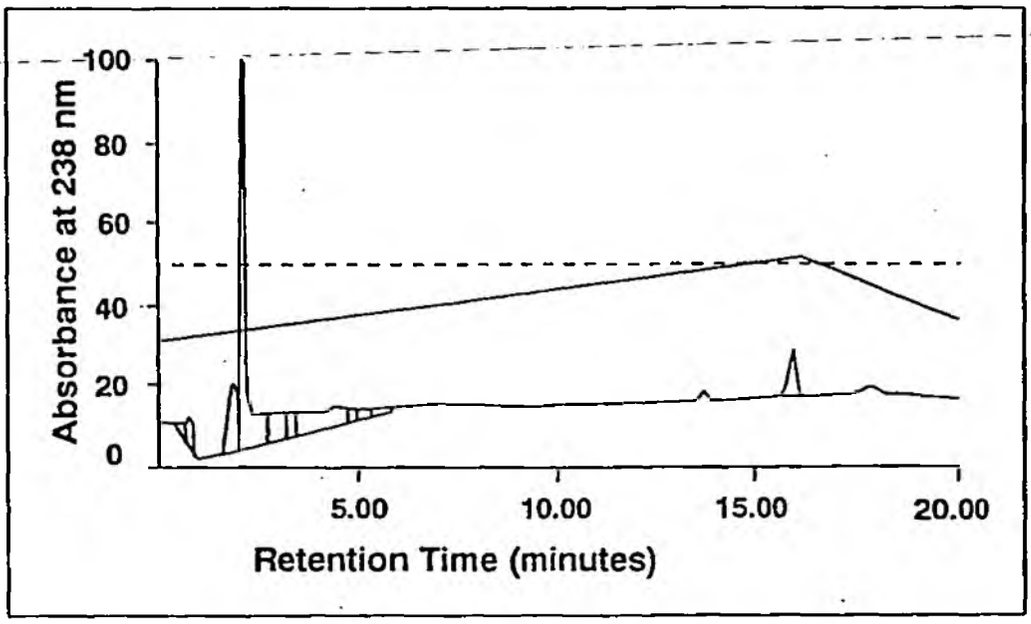

Figure 3.3 HPLC chromatogram of microcystin-LR

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.