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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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.

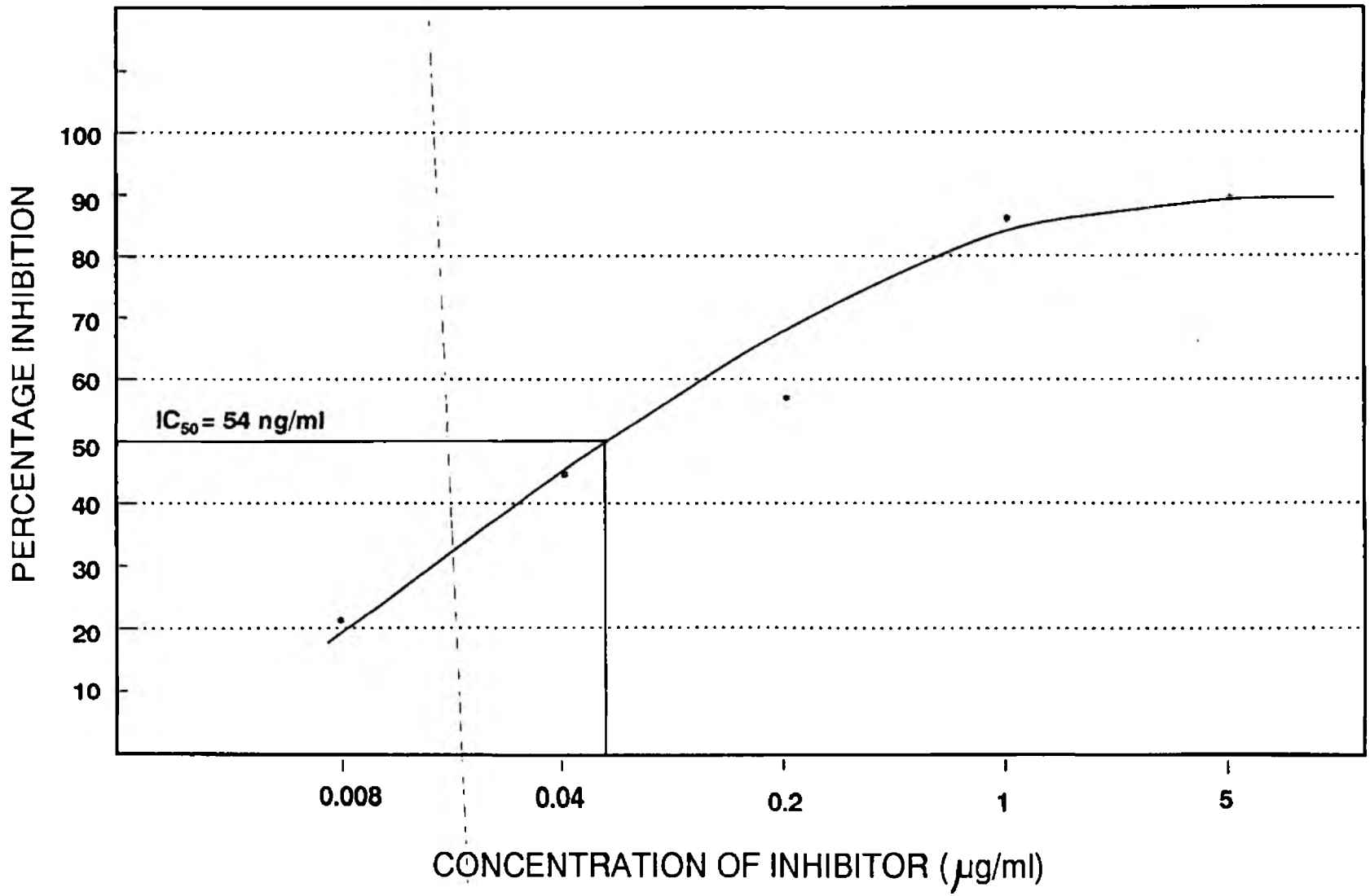
FUSION	CLONE	*IC_{50} (ng/ml)	
		Conjugate coated ELISA plate	Poly-L-lysine activated microcystin-LR coated ELISA plate
A005-1A	15C4	N/A	+0.75
A005-1C/E	9D5	No inhibition	N/A
A005-1D	3D1	No inhibition	No inhibition
A005-1D	4B5	No inhibition	No inhibition
A005-1D	13C6	54	No inhibition
A005-1F	8D2	No inhibition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