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

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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 REVIEW OF TECHNICAL PROGRESS FOR FEBRUARY 1991 - APRIL 1992

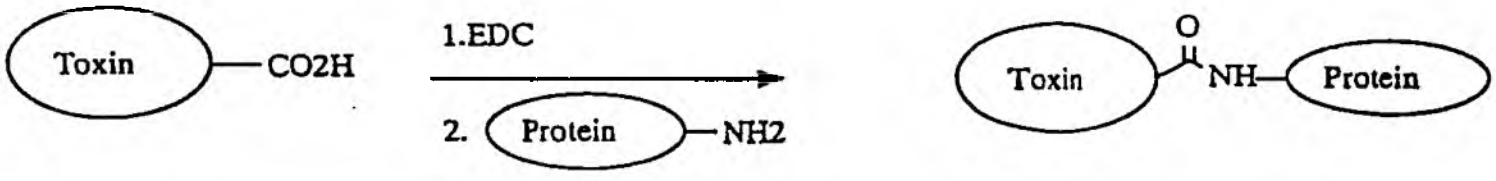
2.1 Preparation of at least two conjugates for testing (Stage 1.1)

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

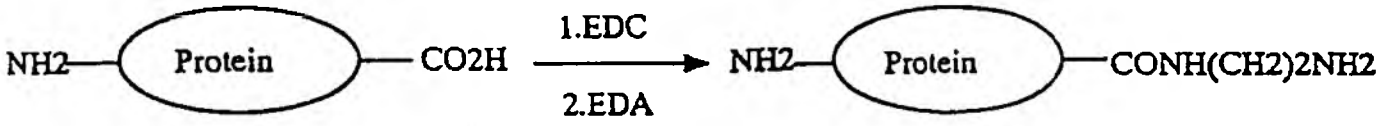


(1) (4a,b,c and d)

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

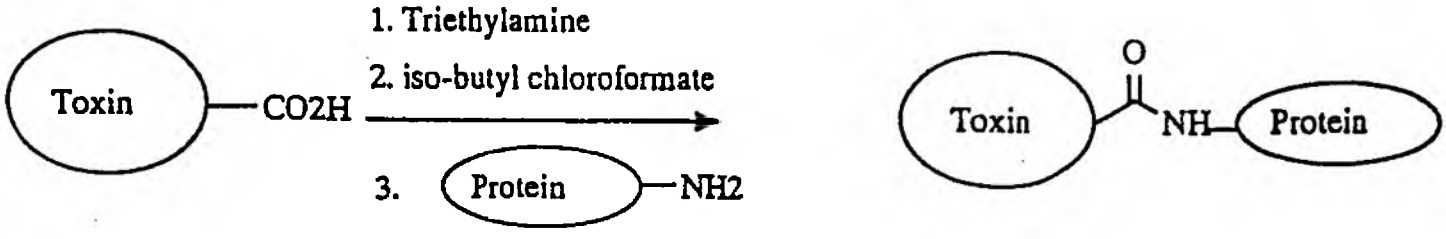


(1) (2a,b)

a; Protein = BSA

b; Protein = C-γ-G

SCHEME 3



(1) (3a,b)

a; Protein = C-γ-G

b; Protein = EDA - C-γ-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	6.1×10^6	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.