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 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.

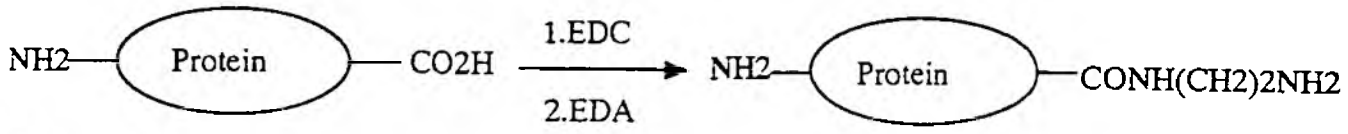
Biocode Limited is in the process of developing on-site testing kits using monoclonal antibodies. These kits are to enable the easy, specific and rapid extraction and identification of microcystin-LR and some structurally related analogues. This work requires the development of novel monoclonal antibodies. 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)

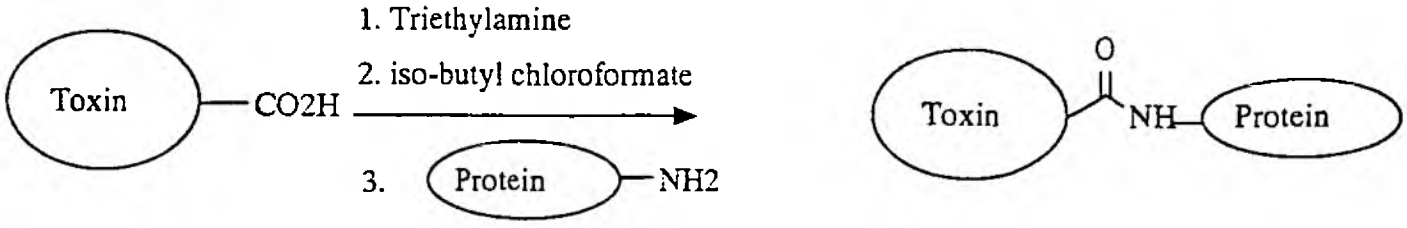
a; Protein = BSA

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)

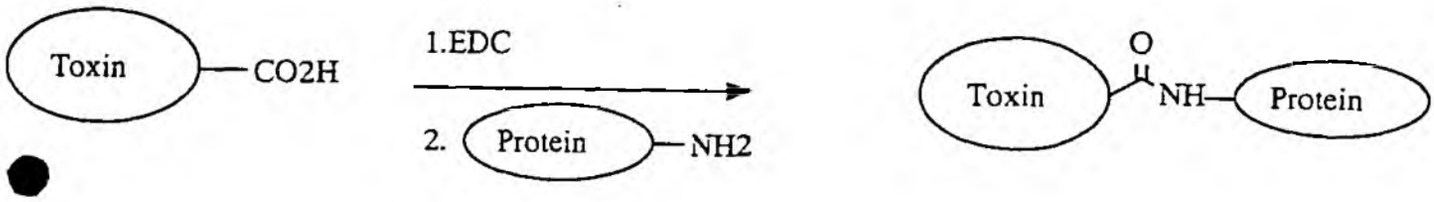
a; Protein = C-ɣ-G

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-X-G
d; Protein = EDA - C-X-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-ϒ-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.