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

ENVIRONMENT AGENCY
135357

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

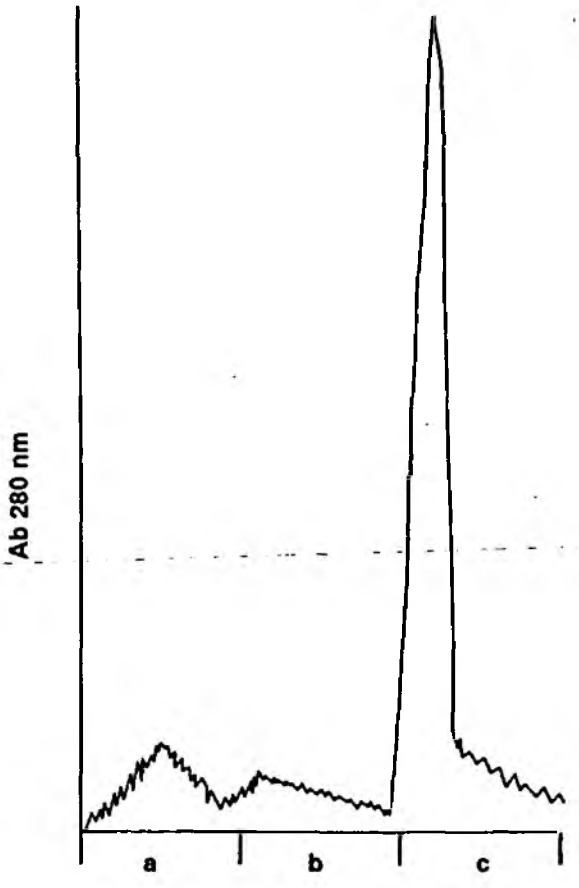
bind significantly to each of the microcystin variants tested.

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.