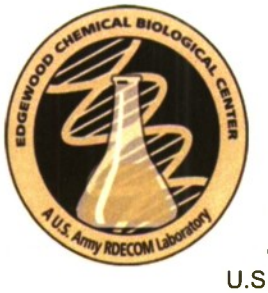

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ECBC-TR-759

PRODUCTION OF MURINE MONOCLONAL ANTIBODIES USING TRADITIONAL AND NOVEL TECHNOLOGY

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March 2010

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
XX-03-2010	Final	Aug 2006 - Nov 2008

4. TITLE AND SUBTITLE

Production of Murine Monoclonal Antibodies using Traditional and Novel Technology

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Dixon, Melissa M. (STC)

5d. PROJECT NUMBER
6007-C08

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
STC, 10 Basil Sawyer Drive, Hampton, VA 23666-1393

8. PERFORMING ORGANIZATION REPORT NUMBER
ECBC-TR-759

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

DIR, ECBC, ATTN: RDCB-DRB-B, APG, MD 21010-5424

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

13. SUPPLEMENTARY NOTES
COR: Jude Height, RDCB-DRB-C, 410 436-4265

14. ABSTRACT

Recent attempts to generate monoclonal antibodies against various biological immunogens have proven to be successful. This report discusses the traditional and novel technologies used to produce monoclonal antibodies. Hybridoma monoclonal antibodies are highly specific antibodies produced in large quantities by cloning a single hybrid cell formed by the fusion of a murine B cell and a tumor cell. Recent attempts to generate monoclonal antibody against Ricin B-Chain and Ovalbumin have proven to be successful. After a pre-determined immunization period, the murine spleens containing the B cells are harvested and fused together with a tumor cell (SP2/0) to generate the first round of monoclonal cells. Subsequent screenings are conducted using the enzyme linked immuno sorbent assay (ELISA) followed by additional cloning steps. The resulting cell lines are stable, monoclonal hybridomas. The supernatant generated from the cell lines are protein purified and tested via ELISA for cross-reactivity. The final product is a hybridoma monoclonal antibody specific to the target for which it was originally immunized

15. SUBJECT TERMS
Hybridoma Murine Monoclonal Antibodies ELISA Ricin B-Chain Ovalbumin

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Sandra J. Johnson
U	U	U	UL	17	19b. TELEPHONE NUMBER *(include area code)* (410) 436-2914

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PREFACE

The work described in this report was authorized under Project No. 6007-C08. The work was started in August 2006 and completed in November 2008.

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Acknowledgments

The author would like to acknowledge Dr. Bonnie J. Woffenden (Science and Technology Corporation [STC]) for her technical advice; Vanessa Funk (STC) for her technical support; Amanda Chambers (U.S. Army Edgewood Chemical Biological Center) for her contribution of the fowlpox virus; Chris Maragos (United States Department of Agriculture) for his contribution of T2-BSA and T2-OVA; Lindsey Miranda and Meredity Moyer (U.S. Army Medical Research Institute of Chemical Defense) for their technical support; and Dr. Mark Poli (U. S. Army Medical Research Institute for Infectious Diseases).

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PRODUCTION OF MURINE MONOCLONAL ANTIBODIES
USING TRADITIONAL AND NOVEL TECHNOLOGY

1. INTRODUCTION

Hybridoma monoclonal antibodies are highly specific antibodies produced in large quantities by cloning a single hybrid cell formed by the fusion of a murine B cell and a tumor cell. Recent attempts to generate a monoclonal antibody against ricin have proven to be successful. Because ricin as a whole is extremely toxic, the B chain, which is non-toxic, was chosen to immunize the mice. The B chain selectively binds to residual groups of galactose present on membrane surfaces found within murine cells. It acts to induce the enclosure of ricin molecules in the B cells.

After a pre-determined immunization period, the murine spleens containing the B cells are harvested and fused together with a tumor cell (SP2/0) to gencrate the first round of monoclonal cells. Subsequent screenings are conducted using the enzyme linked immuno sorbent assay (ELISA) followed by additional cloning steps. The resulting cell lines are stable, monoclonal hybridomas.

The supernatant generated from the cell lines are protein purified and tested via ELISA for cross-reactivity and specificity. The final product is a hybridoma monoclonal antibody specific to the target for which it was originally immunized against.

Monoclonal antibodies currently produced and stored at the U.S. Army Edgewood Chemical Biological Center (ECBC) include Ricin B Chain, OVA, Abrin, Cholera Toxin B, Fowlpox, T-2 ,and Microcystin.

2. MATERIALS AND METHODS

2.1 Antigen

- ***Ricin Toxin B Chain***
  Lyophilized RTB (1.0 mg: Vector) rehydrated in 1 mL phosphate buffered saline (PBS)

- ***Ovalbumin***
  Powder OVA (2.0 mg: Sigma) rehydrated in 1 mL PBS

- ***Abrin Toxin***
  Lyophilized Abrin Toxin (1.0 mg: Toxin Technology Inc.) rehydrated in 1 mL PBS

- ***Abrin Toxoid***
  Lyophilized Abrin Toxoid (1.0 mg: Toxin Technology Inc.) rehydrated in 1 mL PBS

- ***Cholera Toxin B***
  Lyophilized CTB (1.0 mg: Sigma) rehydrated in 1 mL deionized water

- ***Fowlpox***
  Provided by ECBC colleague

- ***T2-OVA***
  Lyophilized T2-OVA (2.0 mg: Chris Maragos USDA) rehydrated in 0.5 mL deionized water

- ***T2-BSA***
  Lyophilized T2-BSA (2.0 mg: Chris Maragos USDA) rehydrated in 0.5 mL deionized water

- ***Microcystin LR-BSA***
  Lyophilized Microcystin LR-BSA (2.0 mg: Mark Poli USAMRIID) rehydrated in 10.25 mL PBS

- ***Microcystin LR-KLH***
  Lyophilized Microcystin LR-KLH (2.0 mg: Mark Poli USAMRIID) rehydrated in 7.5 mL PBS

2.2 Immunization

Female BALB/c mice were injected subcutaneously. Freund's incomplete adjuvant was always used except for the first injection when the antigen was emulsified in Freund's complete adjuvant.

2.3 Myeloma

The SP2/O-Ag 14 (SP2/O) myeloma cells were grown in ISCOVE Modified Eagle Medium (IMDM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% Penicillin/ Streptomycin, 1mM Sodium Pyruvate, and 2mM L-Glutamine, at 37 °C in a 5.0% CO_2 water jacketed incubator.

2.4 Traditional Fusion

The SP2/O cells in log phase of growth were harvested and pelleted via centrifugation at 3500 rpm for 5 min at room temperature. The cells were washed three times with RPMI-1640. The spleens were removed aseptically, and the cells were released by pressing the blunt end of a syringe plunger against a sterile petri dish. Debris was allowed to settle out of solution, and the cells were removed and placed into a fresh, sterile 50 mL conical tube. The red blood cells were lysed with sterile water and RPMI-1640. The suspension was centrifuged at 3500 rpm for 5 min, and the splenocytes were pelleted. The cells were washed three times with RPMI-1640.

The SP2/O and splenocytes were counted and mixed at a ratio of 1:3 to 1:7 and were pelleted by centrifugation. Polyethylene glycol 1500 was added to the cells and gently stirred. The suspension was then diluted with RPMI-1640 and centrifuged at 3500 rpm for 5 min.

The cells were distributed into 96-well tissue culture plates in IMDM containing hypoxanthine, aminopterin, thymidine (IMDM-HAT) plus 1% Penicillin/Streptomycin, 2mM L-Glutamine, 1mM Sodium Pyruvate, and 10% Bovine Growth Serum (BGS). The cells were kept at 37 °C in a 5% CO_2 water jacketed incubator.

2.5 Traditional Screening

All 96-well tissue culture plates were then assayed via ELISA 14 days after fusion. Polystyrene 96-well microtitre ELISA plates were coated with 100 µL of PBS containing 1 to 10 µg of the desired antigen and placed in a 4 °C refrigerator for 12-24 h. After four washes with 10X PBS and 10 mL Tween 20 (ELISA wash buffer), the plates were blocked for 1 h in a 37 °C incubator with 200 µL of 50 g skim milk dissolved in 1X PBS. After four washes with ELISA wash buffer, the plates were incubated with 100 µL of hybrid supernatants for 1 h at 37 °C. After four washes with ELISA wash buffer, 100 µL of anti-mouse IgA + IgG + IgM (H+L) antibody conjugated with horseradish Peroxidase (HRP) (KPL) (1:3000 dilution in 50 g skim milk dissolved in 1X PBS and 1mL Tween 20) were added and incubated at 37 °C for 1 h. After four washes with ELISA wash buffer, 100 µL of substrate solution (1:1 dilution of ABTS Peroxidase Substrate Solution A and Peroxidase Substrate Solution B) were added and incubated for 30 min at 37 °C. The absorbances were read at 405 nm (A_{405} nm) with an ELx800™ Absorbance Microplate Reader (BioTek) (see Table 1).

Table 1. Typical ELISA Values during Screening Procedure

RTB001 Last limiting dilution - 1/64

0.129	0.100	0.183	0.110	0.092	0.097	1.808	0.176	0.084	0.088	0.108	0.105
0.091	0.129	0.177	0.085	0.074	0.069	0.082	0.077	0.096	0.111	0.084	0.083
0.123	2.037	0.091	0.089	0.075	0.083	0.094	0.079	0.077	0.213	0.091	1.734
0.088	0.092	0.187	0.073	0.079	0.069	0.055	0.133	0.070	1.863	1.892	0.096
0.084	0.092	0.087	1.852	0.097	0.075	0.081	0.063	0.072	0.075	0.080	0.088
0.100	1.998	2.100	0.085	0.071	0.149	0.073	0.080	2.068	2.020	0.081	2.064
0.100	0.108	0.319	0.109	0.129	0.120	0.104	1.905	0.097	0.119	0.092	0.178
0.104	0.320	0.114	0.115	1.897	0.143	0.142	0.099	0.118	0.119	0.093	0.085

2.6 Traditional Cloning

The positive hybrids were subcloned once by the limiting dilution procedure in IMDM containing hypoxanthine and thymidine (IMDM-HT) plus 1% Penicillin/Streptomycin, 1mM Sodium Pyruvate, 2mM L-Glutamine and 10% Bovine Growth Serum (BGS) to ensure monoclonality and stability of the cells. Clones were expanded by sequential transfer into 24-well tissue culture plate, 25 and 75 mL tissue culture flasks. The clones were frozen and stored at -195 °C.

2.7 Novel ClonaCell® - HY Fusion

The SP2/O cells were harvested, counted, and resuspended at 2 x 10^7 viable cells. The splenocytes were obtained from a single spleen, counted, and resuspended at 1 x 10^8 viable cells. The 2 x 10^7 SP2/O cells and 1 x 10^8 splenocytes were combined in a 50 mL conical tube and centrifuged for 10 min at ~1350 rpm and pelleted. Polyethylene glycol (PEG) was added to the cells and gently stirred. The suspension was then diluted with Medium B and incubated for 15 min. Medium A was slowly added to the suspension and centrifuged at ~1350 rpm for 7 min to form a pellet. The pellet was resuspended in 10 mL Medium C and transferred to a T'75cm^2 tissue culture flask containing 40 mL of Medium C. The fused cell culture was incubated for 16-24 h at 37 °C in a 5% CO_2 water jacketed incubator.

2.8 Novel ClonaCell® - HY Selection and Cloning

The 16-24 h post fusion transferred the fused cell suspension to a 50 mL conical tube and centrifuge for 10 min at ~1350 to form a pellet. The pellet was resuspended in 10 mL Medium C. The 10 mL cell suspension was transferred into 90 mL of Medium D and mixed completely by inverting the bottle several times. The suspension sat for 15 min at room temperature, allowing the bubbles to rise to the surface. 9.5 mL of the cell suspension were then aseptically plate out into 10 individual 100 mm petri plates. The plates were tilted to level the mixture. The plates were incubated for 14 days at 37 °C in a 5% CO_2 water jacketed incubator.

2.9 Novel ClonaCell® - HY Harvest

At 14 days post fusion; the plates were examined for visible colonies. The isolated colonies were removed from the plates and placed into individual wells of a 96-well tissue culture plate containing 200 µL of Medium E. the 96-well tissue culture plates were incubated at 37 °C, 5% CO_2 for 4 days without feeding.

2.10 Novel ClonaCell® - HY Screening

The same screening procedure was followed as in Section 2.5. Positive responses were transferred into 2 wells of a 24-well tissue culture plate, 100 µL each into 1 mL of Medium E. Once the cells reached a density of 4 x 10^5 cells/mL, one well was frozen and the other was transferred to a T'25cm^2 tissue culture flask with 5 mL of Medium A and 5 mL of Medium E. After the cells reached a density of 4 x 10^5 cells/mL, the culture was transfer to a T'75cm2 tissue culture flask containing 30 mL of Medium A.

2.11 Traditional Additional Cloning Step

An additional "traditional" cloning step was added to verify the stability of the clones. The positive hybrids from Section 2.10 were subcloned once by the limiting dilution procedure in IMDM-HT plus 1% Penicillin/Streptomycin, 1mM Sodium Pyruvate, 2mM L-Glutamine, and 10% BGS to ensure monoclonality and stability of the cells. Clones were expanded by sequential transfer into 24-well tissue culture plates, 25 and 75 mL tissue culture flasks. The clones were frozen and stored at -195 °C.

3. RESULTS AND DISCUSSION

Thirteen hybridomas producing mAbs to various immunogens were selected on the basis of consistently producing positive ELISA results and excreting the desired IgG_1 (κ) isotype (Table 2). These hybridomas were cloned once and expanded. All 13 of these antibodies belonged to the IgG subclass.

Table 2. Characterization of mAbs

mAb	IgG subclass	mAb	IgG subclass	mAb	IgG subclass
RTB001	IgG_1 (κ)	OVA001	IgG_1 (κ)	CTB001	IgG_1 (κ)
RTB002	IgG_1 (κ)	OVA002	IgG_1 (κ)	CTB002	IgG_1 (κ)
RTB003	IgG_1 (κ)	OVA003	IgG_1 (κ)	CTB004	IgG_1 (κ)
		OVA004	IgG_1 (κ)	CTB005	IgG_1 (κ)
		OVA005	IgG_1 (κ)		
		OVA006	IgG_1 (κ)		

4. TROUBLESHOOTING

4.1 Ricin B-Chain and Ovalbumin

The positive hybrids were subcloned once by the limiting dilution procedure in IMDM-HT plus 1% Penicillin/Streptomycin, 1mM Sodium Pyruvate, 2mM L-Glutamine and 10% BGS to ensure monoclonality, and stability of the cells. Clones were expanded by sequential transfer into 24-well tissue culture plates, 25 and 75 mL tissue culture flasks. The clones were frozen and stored at -195 °C.

The positive hybrids were expanded for protein purification, where it was determined the clones were not monoclonal at the time of the initial selection and freezing. To correct this problem, all positive hybrids were expanded and subcloned two additional times by the limiting dilution procedure that was modified from 3 cells per well (cpw), 1 cpw, ½ cpw and ¼ cpw to ¼ cpw, 1/16 cpw, 1/32 cpw and 1/64 cpw.

By applying the probability theory, Poisson distribution (Tables 3 and 4), we were able to calculate with certain confidence the clones have a high probability of being monoclonal. Lower probability = Higher confidence in monoclonality.

Clones were expanded by sequential transfer into 24-well tissue culture plates, 25 and 75 mL tissue culture flasks. The clones were frozen and stored at -195 °C.

4.2 Cholera Toxin B

The positive hybrids were subcloned twice by the limiting dilution procedure in IMDM-HT plus 1% Penicillin/Streptomycin, 1mM Sodium Pyruvate, 2mM L-Glutamine and 10% BGS to ensure monoclonality, and stability of the cells. Clones were expanded by sequential transfer into 24-well tissue culture plates, 25 and 75 mL tissue culture flasks. The clones were frozen and stored at -195 °C.

Table 3. Probability Theory – Poisson Distribution 37% or Less Positive Clones
RTB002 Last Limiting Dilution - 1/64

0.139	0.161	0.140	0.134	1.735	0.096	0.261	0.147	0.108	0.475	0.089	1.782
0.116	0.104	0.104	0.228	0.191	1.671	0.271	0.168	0.173	0.232	0.107	0.099
0.158	0.142	0.140	1.641	0.298	0.111	0.260	0.695	0.306	1.653	0.223	0.092
0.084	0.096	0.160	0.196	1.931	0.202	0.097	0.221	0.288	0.267	0.239	0.186
2.002	0.083	0.136	0.215	2.101	0.132	0.149	0.099	0.155	0.240	0.119	0.107
1.836	0.118	0.265	0.220	0.184	1.436	0.144	0.085	1.907	0.375	1.316	0.342
1.772	1.786	1.381	0.083	0.086	0.093	0.069	0.165	0.117	0.087	0.077	0.623
0.128	0.115	1.902	0.128	0.103	0.119	0.081	0.101	0.088	0.715	0.087	2.320

H12 = parent clone, D5 and E1 = sister clones

Control Set-up	Control Values						Control Average
Coating + 1° Antibody	1.571	1.398	1.377	1.291	1.395	1.245	1.380
No Coating + No 1° Antibody	0.108	0.091	0.151	0.101	0.100	0.096	0.108

(-) Control Set-up	(-) Control Average Values	(-) Control Average
No Coating + No 1° Antibody	0.108	0.108

3X (-) Control Calculation	37% or Less (+) Clones	Monoclonality Probability
0.108 x 3 = 0.324	23 (+) Clones / 96 wells = 0.239	**23.95% chance**
(+) Clones of 0.324 or higher will be selected	0.239 x 100 = 23.95%	**clones are monoclonal**

Table 4. Probability Theory - Poisson Distribution 37% or Less Positive Clones
OVA005 Last Limiting Dilution – 1/64

0.277	0.250	0.778	0.895	0.919	0.784	0.355	0.437	2.350	0.340	0.337	0.416
0.213	0.677	0.824	0.631	0.640	0.424	0.465	0.423	0.570	0.377	0.387	0.223
2.475	0.335	0.528	0.496	0.562	0.529	1.837	0.377	0.382	0.322	0.364	0.255
0.350	0.320	0.268	0.162	0.113	0.203	0.174	0.465	1.816	0.374	0.483	0.273
2.471	0.393	0.531	0.318	0.349	0.280	1.875	0.128	0.102	0.328	0.333	0.238
0.316	0.363	0.626	0.321	0.311	0.233	0.121	0.242	0.367	0.271	0.334	0.190
0.317	0.266	0.536	0.337	2.167	2.113	0.287	0.166	2.293	0.319	0.368	0.153
0.272	0.284	0.279	0.277	1.018	0.354	0.294	0.322	0.288	0.418	0.233	0.165

C1 = parent clone, E1 and A9 = sister clones

Control Set-up	Control Values				Control Averages
Coating + 1° Antibody	2.648	2.534	2.542	2.640	2.591
Coating + No 1° Antibody	0.134	0.136	0.108	0.168	0.137
No coating + 1° Antibody	0.147	0.172	0.125	0.125	0.142
No coating + No 1° Antibody	0.120	0.143	0.189	0.132	0.146

(-) Control Set-up	(-) Control Average Values	(-) Control Average
Coating + No 1° Antibody	0.137	
No coating + 1° Antibody	0.142	
No coating + No 1° Antibody	0.146	0.142

3X (-) Control Calculation	37% or Less (+) Clones	Monoclonal Probability
	30 (+) clones / 96 wells =	
0.142 x 3 = 0.426	0.3125	**31.25% chance**
(+) Clones of 0.426 or higher will be selected	0.3125 x 100 = 31.25%	**clones are monoclonal**

The positive hybrids were expanded for protein purification where it was determined the clones were not monoclonal at the time of the initial selection and freezing. To correct this problem, all positive hybrids were expanded and subcloned an additional time by the limiting dilution procedure, which was now adopted from the Ricin-B Chain and Ovalbumin troubleshooting. The limiting dilution procedure was slightly modified to account for the rapidly growing cells. The 1/32 cpw was removed and 1/256 cpw was added.

By applying the probability theory, Poisson distribution, (Table 5), we were able to calculate with certain confidence the clones have a high probability of being monoclonal. Lower probability = Higher confidence in monoclonality.

Clones were expanded by sequential transfer into 24-well tissue culture plates, 25 and 75 mL tissue culture flasks. The clones were frozen and stored at -195 °C.

Table 5. Probability Theory - Poisson Distribution 37% or Less Positive Clones
CTB004 Last Limiting Dilution – 1/256

0.206	0.329	0.294	0.301	0.274	2.614	0.366	0.305	0.310	0.312	0.213	1.252
0.787	0.281	0.371	0.329	0.087	0.337	0.287	0.277	0.278	0.274	0.262	0.638
0.319	0.254	0.355	0.315	0.345	0.091	2.296	0.343	2.373	0.360	0.284	0.308
0.325	0.339	0.378	0.357	0.076	0.086	0.099	0.082	0.183	0.312	0.316	0.326
0.327	0.368	0.322	0.180	0.083	0.085	0.080	0.280	0.076	0.287	0.343	2.873
0.313	0.256	1.267	0.396	2.481	0.091	0.098	0.290	0.262	0.296	0.280	2.871
0.281	0.299	0.336	0.292	0.316	0.340	0.113	0.323	0.302	0.292	0.271	0.620
0.134	0.270	0.395	0.304	0.279	0.242	0.272	0.277	0.295	0.250	0.274	0.273

** E12 = parent clone, F12 and A6 = sister clones**

Control Set-up	Control Values				Control Averages
Coating + 1° Antibody	2.727	2.511	2.527	2.607	2.593
Coating + No 1° Antibody	0.104	0.170	0.083	0.091	0.112
No coating + 1° Antibody	0.160	0.125	0.092	0.096	0.118
No coating + No 1° Antibody	0.100	0.240	0.107	0.131	0.145

(-) Control Set-up	(-) Control Average Values	(-) Control Average
Coating + No 1° Antibody	0.112	
No coating + 1° Antibody	0.118	
No coating + No 1° Antibody	0.145	0.125

3X (-) Control Calculation	37% or Less (+) Clones	Monoclonal Probability
	14 (+) clones / 96 wells = 0.1458	
0.125 x 3 = 0.375		**14.58% chance**
(+) Clones of 0.375 or higher will be selected	0.146 x 100 = 14.58%	**clones are monoclonal**

4.3 Abrin Toxin and Toxoid

The positive hybrids were subcloned twice by the limiting dilution procedure in IMDM-HT plus 1% Penicillin/Streptomycin, 1mM Sodium Pyruvate, 2mM L-Glutamine and 10% BGS to ensure monoclonality, and stability of the cells. Clones were expanded by sequential transfer into 24-well tissue culture plates, 25 and 75 mL tissue culture flasks. The clones were frozen and stored at -195 °C.

The supernatant from the positive hybrids were purified to determine if the clones were monoclonal or needed to undergo further selection.

The cell lines produced were extremely slow growing but stable.

4.4 Fowlpox Virus

Due to the lack of a positive control antibody at the time of the fusion, the fusion was performed and at 14 days post fusion, the cells were harvested and frozen at -195 °C. A positive control antibody was subsequently obtained and tested on the immunogen. The results were disappointing and indicated a failure. It is believed that the storage of the virus was improper, which lead to the lack of an immune response in the mice. Further testing has been postponed at this time.

4.5 T2-OVA and T2-BSA

The T2-OVA cells did not grow and were subsequently deemed a failure. Further testing of T2-OVA has been postponed at this time.

The T2-BSA positive hybrids were subcloned twice by the limiting dilution procedure in IMDM-HT plus 1% Penicillin/Streptomycin, 1mM Sodium Pyruvate, 2mM L-Glutamine and 10% BGS to ensure monoclonality, and stability of the cells. Clones were expanded by sequential transfer into 24-well tissue culture plates, 25 and 75 mL tissue culture flasks. The clones were frozen and stored at -195 °C.

The supernatant from the positive hybrids will be purified to determine if the clones are monoclonal or need to undergo further selection.

The cell lines produced are extremely slow growing but stable.

4.6 Microcystin LR-BSA

A new fusion protocol was tested to see if monoclonality could be reached at a quicker rate and still maintain the same quality product. The ClonaCell® -HY Hybridoma Cloning Kit from StemCell Technologies (Vancouver, BC) was tested and proven to be successful.

The supernatant from the positive hybrids will be purified so that characterization and down selection can commence.

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