AD_____

Award Number: DAMD17-02-1-0659

TITLE: Process Research and Development of Antibodies as Countermeasures for *C. botulinum*

PRINCIPAL INVESTIGATOR: Michael Meagher, Ph.D.

CONTRACTING ORGANIZATION: University of Nebraska, Lincoln
Lincoln, Nebraska 68588-0430

REPORT DATE: March 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20040524 137

# REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | March 2004 | Annual (1 Mar 2003 - 28 Feb 2004) |

**4. TITLE AND SUBTITLE**
Process Research and Development of Antibodies as Countermeasures for *C. botulinum*

**5. FUNDING NUMBERS**
DAMD17-02-1-0659

**6. AUTHOR(S)**
Michael Meagher, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
University of Nebraska, Lincoln
Lincoln, Nebraska 68588-0430

*E-Mail:* mmeagher1@unl.edu

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT (Maximum 200 Words)**

This report describes the project to fill out shell space on the third floor of Othmer Hall, the new home for the University of Nebraska-Lincoln Biological Process Development Facility. Construction is 90% completed and all research laboratories of the BPDF will be functional in May 2004. Detailed design of the cGMP basement has been initiated and is expected to be completed by Fall 2004. The third floor will house all of the process research capabilities, i.e. molecular biology, fermentation, cell culture, analytical methods, quality control, purification development and media prep.
This report also describes research on the expression of antibodies in Chinese Hamster Ovary (CHO) cells against serotype A botulinum neurotoxin. We have been able to clone and successful express the full intact S-25 antibody provided by Dr. Jim Marks of UCSF at 30 mg/L in 250 mL spinner shake flasks. A manuscript has been submitted on this work.

**14. SUBJECT TERMS**

**15. NUMBER OF PAGES**
38

**16. PRICE CODE**

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

## Table of Contents

Cover.....1

SF 298.....2

Table of Contents.....3

Introduction.....4

Body.....4

References.....19

Key Research Accomplishments.....38

## Introduction

The funding for this project is comprised of two components. The first component, which represents 95% of the funding, is dedicated to filling out shell space on the third floor and basement of Othmer Hall, the new home for the Biological Process Development Facility. The other 5% is dedicated to research focused on the expression of antibodies against the botulinum neurotoxins in Chinese Hamster Ovary (CHO) cells. This report will provide a summary of 2003-2004 activities, which includes construction of third floor research space and expression of a chimeric full-length antibody against serotype A. UNL just submitted a manuscript (4/15/04) on the BoNTA chimeric antibody project, which is incorporated into this report.

## Body

### Construction Project

***Basement cGMP Construction Project***

On December 2003 the University of Nebraska Board of Regents officially approved the cGMP basement project. A competitive bid process was initiated in February 2004 for detailed design of the cGMP facility. This facility will be a 15,000 $ft^2$ and capable of producing Phase I/II material derived from bacteria, yeast, mammalian cells and transgenic milk. The design firm that was awarded the bid was bioKinetics of Philadelphia, PA and Davis Design of Lincoln, NE. Anticipated completion of detail design is fall 2004. This design document will be used for construction bids.

***Third Floor Construction Project***

The majority of the first year (2002-2003) was spent designing the third floor and basement facility, which are currently unfinished shell space. This was performed through a local architectural firm, Davis Design, and required 9 months to complete. The result of this phase of the project was a set of bid documents. Through a competitive process the contract was awarded to Builder, Inc of Lincoln Nebraska and construction was initiated in March 2003. The third floor of Othmer Hall will house research laboratory and administrative and staff office space. As of April 2004 the project is at 90% completion. It is expected that the Mammalian Cell Culture Laboratories will be completed by May 2004. The delay was a result of a redesign of the cGMP basement facility. The Non-cGMP Pilot Plant that was programmed for the basement was eliminated and incorporated into the third floor design. This was done to provide more space for raw material shipping and receiving for the cGMP facility.

The last 10% of the project represents that Master Cell Banking Facility. Over the last 6 months the design of the facility has been updated to meet the requirements for both the FDA and EC for Biologics. It is expected that the Master Cell Banking Facility will not be completed until Fall 2004.

The third floor will accommodate all of the research capabilities of the BPDF, along with a Class 10,000 laboratory dedicated to production of Master Cell Banks (MCB) and Working Cell Banks (WCB) and two faculty members in the Department of Chemical Engineering The third laboratory is now a part of the BPDF and is occupied by the Analytical Methods Laboratory.. The entire third floor of Othmer Hall is 14,000 $ft^2$ and is dedicated to Bioprocess and Bioengineering research. A description of the third floor labs are described below.

***Molecular Biology (MBL):*** A 990 $ft^2$ laboratory dedicated molecular biology and strain construction.

***Fermentation Development Laboratory (FDL):*** A 1,200 $ft^2$ laboratory dedicated to bacterial and yeast fermentation research. The FDL will have 10 autoclavable BioFlo III and Bioflo 3000 bench-top 5 L fermentors (New Bruinswick Scientific) and 8 Bioengineering NLF 19 L steam-in-place fermentors. The FDL will have a 150 $ft^2$ microbiology support laboratory for growing inoculums and storing frozen seed cultures. All of the fermentors will be computer controlled and off-gas will be sent to an off-gas mass spectrometer for metabolic analysis.

***Cell Culture Development Laboratory (CCDL):*** The CCDL is a new capability for the BPDF and has two different laboratories. One half of the space (750 $ft^2$) is dedicated to mammalian cell-line development and optimization and is outfitted with biosafety cabinets and $CO_2$ incubators. The purpose of this space is to develop mammalian cell clones suitable for transfer into a bioreactor and process scale-up. The other half of the facility (750 $ft^2$) is dedicated to bioreactors for scaling-up the process from 250 mL spinners to a 200 L bioreactor. The CCDL will have 8 by 250 mL DasGip computer controlled spinner system, 4 by 3.7 L computer controlled Bioengineering ALF bioreactors integrated to a Nova 400 bioanalyzer for automated sample analysis, one 19 L computer controlled Bioengineering bioreactor and

one 200 L computer controlled Bioengineering bioreactor. The off-gas from the 3.7, 19 and 200 L bioreactors will be sent to an off-gas mass spectrometer. The primary function of the CCDL will be strain development, process research and development of production technology for humanized antibodies against bioterrorism agents in CHO cells.

***Purification Development Laboratory (PDL)*:** The PDL is a 1,500 $ft^2$ laboratory that has the responsibility of developing the recovery and purification process at the bench-scale (7.8 to 100 mL column size) and scaling-up the process to the 1 to 2 L column size and the production of small non-cGMP lots. The PDL will have "three research areas," bench-scale development area, central bench for routine assays, and the third is a small pilot plant area capable of processing up to 0. 5 to 1 gram research lots of material.

***Media Preparation Area*:** The third floor will have a central media preparation area that will have two 26" x 36" x 38" Primus Autoclaves and a Miele Model G7827 large capacity glassware washer. There are two small lab areas will include a chemical hood, chemical storage cabinets and dry goods storage and laboratory space to prepare buffer and media.

***Dark Room and Radioisotope Room.*** Two small 150 $ft^2$ labs are designated as a dark room and a radioisotope room.

***cGMP Master Cell Banking Suite.*** A 350 $ft^2$ laboratory has been dedicated to cGMP production of Master Cell Banks (MCB) and Working Cell Banks (WCB). This suite will have approximately 360 $ft^2$ of Class 10,000 space with a 90 $ft^2$ Class 100,000 ante room for gowning. There will be a 600 ft Class 100,000 cGLP microbiology support laboratory which will have a 26" x 26" x 38" cGMP pass-thru autoclave and will provide all of the testing necessary to support MCB and WCB production. This GLP microbiology laboratory will also provide QC microbiology support for the cGMP pilot plants in the basement of Othmer Hall.

*QC Microbiology Laboratory:* Adjacent to the cGMP MCB facility is the QC microbiology laboratory. This lab will occupy approximately 600 $ft^2$. The purpose of this laboratory is to provide microbiology support the development laboratories and the cGMP facility.

***Three Bioengineering Research Laboratories.*** Three standard research laboratories are being constructed on the third floor. These lab spaces are planned for two faculty members in the Department of Chemical and the third laboratory will be used for the BPDF Analytical Methods Laboratory. One faculty member is working on antibody production and tissue engineering, while the second faculty member research interest is the production of complex proteins in transgenic animal. (Note: All animals for transgenic research will be housed in an animal facility in either the Departments of Animal Science or Veterinary and Biomedical Sciences on East Campus at the University of Nebraska-Lincoln.

***Third Floor Office Area.*** The third floor will have office area for both staff and students. There will be an office area for the BPDF staff, which includes a conference room with both audio and visual conferencing capabilities.

***Additional Features of the Third Floor***

***Utilities.*** The third floor will have all of the standard utilities and additional utilities that are specific to the third floor. These include chilled water (10°C), clean steam distributed in 316L stainless steel piping, a biowaste kill system that will serve both the third floor and the basement, central gas storage room for distribution of oxygen, nitrogen, carbon dioxide and helium.

***Electrical.*** The third floor will have 3 different types of power, uninterruptible power supply (UPS), emergency power (EP), and normal. The UPS system is located on the third floor electrical closet and is designated for critical systems, such as bioreactors, critical computer systems, and critical analytical equipment. The EP system is designed to come on 10 seconds after a power outage. Critical systems such as cold rooms, refrigerators, freezers, and equipment that can withstand a 10 second outage and still function will be on this system.

***Security.*** The third floor will have a security system from AMAG Access Control Systems, the AMAG 625/675. This system will include electronic proximity detectors for all doors, video surveillance on all corridors, controlled access to the third floor, and an alarm system with motion sensors that are connected to the local police department which will indicate the location and time of unauthorized entrance or motion.

Monoclonal Antibody Research Project

## Submitted Manuscript

# PRODUCTION AND PURIFICATION OF A CHIMERIC MONOCLONAL ANTIBODY AGAINST BOTULINUM NEUROTOXIN SEROTYPE A

**Mark C. Mowry*, Mike Meagher*, Leonard Smith[+], Jim Marks[x], and Anu Subramanian***

* Department of Chemical Engineering, University of Nebraska, 207 Othmer Hall, 820 North 16th Street, Lincoln, NE 68588

[+] Toxinology and Aerobiology Division, U.S. Army Medical Research Institute of Infectious Diseases, Frederic, MD 21702

[x] Department of Anesthesia and Pharmaceutical Chemistry, University of California, Room 3C-38, San Francisco General Hospital, 1001 Potrero Avenue, San Francisco, CA 94110

Corresponding Author: Mark C. Mowry, Department of Chemical Engineering, University of Nebraska, 207 Othmer Hall, 820 North 16th Street, Lincoln, NE 68588

Email: mmowry2@unl.edu

Tel: (402) 472-2752 Fax: (402) 472-6989

**ABSTRACT**

Production of recombinant antibodies against botulinum neurotoxin is necessary for the development of a post-exposure treatment. CHO-DG44 cells were transfected with a plasmid encoding the light and heavy chains of a chimeric monoclonal antibody (S25) against botulism neurotoxin serotype A. Stable cell lines were obtained by dilution cloning and clones were shown to produce nearly equivalent levels of light and heavy chain antibody by an enzyme-linked immunosorbent assay (ELISA). In suspension culture, cells produced 35 μg/ml of chimeric antibody after 6 days, corresponding to a specific antibody productivity of 3.1 pg/cell/day. A method for the harvest and recovery of an antibody against botulism neurotoxin serotype A was investigated utilizing ethylenediamine-N,N'-tetra(methylphosphonic) acid (EDTPA) modified zirconia and MEP-hypercel, a hydrophobic charge interaction chromatography resin. Purification of the S25 antibody was compared to that achieved using rProtein A Sepharose Fast Flow resin. After the direct load of culture supernatant, analysis by ELISA and gel electrophoresis showed that S25 antibody could be recovered at purities of 41.3% and 43.7%, from the EDTPA modified zirconia and MEP-hypercel columns, respectively. Although the purity obtained from each of these columns was low, the ability to withstand high column pressures and nearly 90% recovery of the antibody makes EDTPA modified zirconia well suited as an initial capture step. Combining the EDTPA modified zirconia and HCIC columns in series resulted in a purity of 71.6% and a final product yield of 71.9%.

## INTRODUCTION

An increasing number of recombinant monoclonal antibodies are being developed for the treatment of medical conditions such as cancer, arthritis and autoimmune diseases [1,2]. To meet the increased demand for monoclonal antibodies, all aspects of antibody production and purification need to be improved. Another potential use of monoclonal antibodies is the treatment for exposure to toxins, such as botulism neurotoxin (BoNT), one of the most poisonous substances known [3]. BoNT has been classified by the Centers of Disease Control (CDC) as one of the six highest risk threats for use in bioterrorism due to its potency, lethality and ease of production [4]. BoNT is produced by the spore forming bacteria *Clostridium botulinum*, and consists of seven serotypes (A-G) that cause the human disease botulism [5]. Botulism is characterized by flaccid paralysis and often results in death. The paralytic ability of the toxin has led to medical treatments for muscle conditions such as cervical distonias, cerebral palsy, and posttraumatic brain injury, in addition to its use for cosmetic purposes [6].

The potential use of BoNT in bioterrorism requires either a vaccine or other treatment for exposure. Currently, there are no small molecule drugs available to prevent botulism, although a pentavalent toxoid is available from the CDC. In addition to the pentavalent toxoid a recombinant vaccine is being developed [7, 8]. Regardless of the availability of a recombinant vaccine, mass vaccination is unlikely due to the rarity of exposure and the fact the vaccination would prevent medical uses of BoNT. Antibodies, however, can be used for the post-exposure treatment of botulism. Equine antitoxin and human botulism immune globulin have been used for the post-exposure treatment of botulism [9, 10]. Recombinant monoclonal antibodies are currently being developed for the treatment of botulism. Three monoclonal antibodies have been combined to neutralize 450,000 50% lethal doses of BoNT serotype A [3].

Post exposure treatment of botulism would consist of a mixture of monoclonal antibodies against each of the seven BoNT serotypes. To produce large quantities of these recombinant monoclonal antibodies, it is necessary to improve production and purification methods. Several mammalian expression systems have been used for the high level expression of monoclonal antibodies, including the use of dihydrofolate reductase (dhfr) deficient Chinese hamster ovary (CHO) cells [11]. These cells allow for amplification of gene expression upon the addition of methotrexate [12-15]. It has been shown that the productivity of CHO cells increases with gene copy number [16].

Upon selection in medium containing stepwise increases in methotrexate, CHO cells with monoclonal antibody productivities (qAb) as high as 100 pg/cell/day have been obtained [17].

After a production cell line is established, it is necessary to develop techniques for purification of the monoclonal antibody. The most common method of antibody purification is affinity chromatography based on Protein A or Protein G [18-23]. These purification methods are effective, but the sorbent is expensive and the leakage of Protein A results in the need for further purification processes. The expense and harsh elution conditions of affinity sorbents such Protein A has led to the search for alternative purification processes. These include hydrophobic interaction chromatography [24, 25], hydoxylapatite [26] and ion-exchange chromatography [27]. Many of these purification techniques require significant treatment of the culture supernatant prior to purification. We have focused our efforts on purification using two different chromatography resins, MEP-hypercel and ethylenediamine-N,N'-tetra(methylenephosphonic) acid (EDTPA) modified zirconia.

In the present study, we have developed and characterized production of a monoclonal antibody in a dhfr deficient CHO cell line and have analyzed a purification scheme that uses EDTPA modified zirconia as an initial capture and purification step followed by a secondary purification using MEP-hypercel, a hydrophobic charge interaction chromatography (HCIC) resin. EDTPA modified zirconia has previously been used for the separation of antibody from bovine serum albumin, a common component of mammalian cell culture medium [28, 29]. Zirconia based resins provides excellent thermal and chemical stability compared to more typical resins. The zirconia surface is modified with EDTPA to block direct binding of antibody to the zirconia, which can lead to tailed elution bands and irreversible binding [30]. Hydrophobic charge induction chromatography (HCIC) has been used to purify antibodies directly from cell culture supernatant [31]. HCIC takes advantage of the pH behavior of the ionizable ligands. A decrease in the pH causes both the ligand and the protein to become positively charged, overcoming the hydrophobic interactions (Burton and Harding, 1998). To obtain purified antibody against BoNT serotype A, CHO DG44 cells were transfected with the genes for the light and heavy chains of the S25 antibody, and a purification scheme utilizing EDTPA modified zirconia and HCIC was compared to that obtained using a Protein A based resin.

## MATERIALS AND METHODS

### Cell Line, Media, Transfection and Expression Vectors

CHO-DG44 cells, which are dhfr negative, were obtained from Dr. Larry Chasin (Columbia University). This host cell line was maintained in α-MEM media (Invitrogen) supplemented with 8% fetal bovine serum (FBS)(Invitrogen). The pS25 plasmid (Figure 1) was constructed by inserting the chimeric light and heavy chain IgG genes against BoNT serotype A, along with the gene for dhfr into the plasmid pcDNA3.1(+) (Invitrogen).

CHO-DG44 cells were transfected with the pS25 plasmid using Lipofectamine 2000 (Invitrogen). Cells were seeded at 0.5 ml in 24 cell plates at a density of $2 \times 10^5$ cell/ml in α-MEM media containing 8% FBS and grown overnight. One μg plasmid DNA and 0.5-2.0 μl Lipofectamine 2000 were combined in 0.1 ml Opti-MEM media (Invitrogen) and equilibrated for 20 min. Plasmid DNA was added to the transfection mix either uncut or linearized with NruI placing the amplifiable gene (dhfr) between the heavy and light chains, increasing the likelihood that both the light and heavy chains would be amplified upon methotrexate addition. The DNA/Lipofectamine 2000 solution was added to the 24 well plates and the plates were incubated at 37°C overnight. Stably transfected cells were selected in α-MEM media lacking ribonucleotides and deoxyribonucleotides, which prevent cells lacking dhfr from growing. Cells were passed several times and individual clones were obtained by dilution cloning at 0.5 cells/well in 96 well plates.

### ELISA

The concentration of the whole antibody, as well as the concentration of the light and heavy chain portions, was determined using an enzyme-linked immunosorbent assay. Affinity purified rabbit anti-human IgG antibodies were diluted to 5 μg/ml in coating buffer (100 mM $NaHCO_3$, 100 mM NaCl, pH 9.3). One hundred μl diluted antibody was added to 96 well plates (Nunc) and incubated overnight at 4°C. The plates were washed twice with Tris buffer (20 mM Tris-HCl, 50 mM NaCl, pH = 7.2) containing 0.1% Tween 20 and then twice with Tris buffer alone. Blocking buffer (Tris buffer containing 0.5% BSA or casein) was added to the 96 well plates and incubated at 37°C for 1 h. Supernatant samples were diluted in blocking buffer and samples were loaded into the 96 well plates in triplicate. Plates were incubated for 1 h at 37°C and the washing procedure was repeated. One hundred μl of a goat anti-human IgG-HRP conjugate antibody diluted to 0.5 to 2 μg/ml in the dilution buffer was added to the plates.

The plates were incubated for 1 h at 37°C and the washing procedure was repeated. Lastly, 100 μl of 1 mg/ml ABTS in ABTS buffer (Roche) was added to the plates. The absorbance was determined at 405 nm using an ELx800 plate reader (Bio-Tek) after 30 min incubation. This procedure was used for whole antibody, heavy chain (Fc specific), and light chain (κ specific). Whole, Fc and kappa rabbit anti-human IgG coating antibodies and whole, Fc and kappa goat anti-human IgG-HRP conjugated antibodies were used in the ELISAs (Sigma).

**Transfer to Suspension Culture**

After screening the clones for antibody production, nine clones that reached 0.5 μg/ml antibody after three days were transferred to suspension culture. Initially, cells were seeded in the spinner flasks at 2-3 x $10^5$ viable cells/ml in CHO-S-SFMII media (Invitrogen) containing 1% FBS. The cells were then passed every 2-4 days into fresh media containing decreasing amount of FBS. After 8-10 passages the cells were frozen in 1.5 ml aliquots in α-MEM media containing 10% FBS and 10% dimethylsulfoxide (DMSO) at a cell density of $10^7$ cells/ml.

**Growth of Cells in Suspension Culture**

The CHO-DG44 S25 #56 cell line was grown in batch culture to analyze antibody production in suspension culture and to produce a sufficient amount of S25 antibody for purification and analysis. Frozen cells were resuspended in 40 ml CHO-S-SFMII at a seeding density of 3-4 x $10^5$ cells/ml. The spinner flasks were incubated at 37°C and 5% $CO_2$. The cells were fed every 3-4 days for several passages and were then seeded at 2 x $10^5$ viable cell/ml in 350 ml CHO-S-SFMII media in a 1L controlled spinner flask. The dissolved oxygen (DO), pH and temperature were controlled using a DAS-GIP Cellferm-Pro control system (Julich, Germany). The pH was controlled by addition of $CO_2$ and 1M NaOH. Samples were taken every day and viability and cell density were determined by trypan blue exclusion and counting on a hemocytometer. Cell suspensions were centrifuged at 1200 rpm for 5 min and supernatant samples were frozen for later analysis.

**Protein Purification**

EDTPA modified zirconia (Zirchrom), MEP-hypercel (Ciphergen), and rProtein A Sepharose Fast Flow (Pharmacia) resins were compared for the purification of S25 antibody. The supernatant from the CHO-DG44 S25

#56 cells was harvested after 6 days in batch culture. Supernatant was harvested by centrifugation at 300xg for 5 min followed by a 5-fold diafiltration with PBS (20 mM $Na_2HPO_4$, 150 mM NaCl, pH=7.4) using a Pellicon XL50 ultrafiltration device containing 0.005 $m^2$ of a 10 kD MWCO Biomax membrane.

Supernatant samples were purified using an AKTA FPLC (Pharmacia). Diafiltered samples were purified using a 100 mm x 4.6 mm diameter column containing Protein A Sepharose Fast Flow resin (Pharmacia). Alternatively, culture supernatant was directly loaded onto the rProtein A Sepharose Fast Flow column, an MEP-hypercel column (100 mm x 4.6 mm dia.), or an EDTPA modified zirconia column (Zirchrom)(50 mm x 4.6 mm dia.). Prior to loading the EDTPA modified zirconia column, the supernatant was diluted 1:1 in 40 mM MES buffer containing 8 mM EDTPA.

After loading, the columns were washed with 5 column volumes (CV) of equilibration buffer. The rProtein A Sepharose Fast Flow resin was loaded using PBS (pH 7.2) and was eluted in 50 mM sodium citrate (pH-3.0). The MEP-hypercel column was equilibrated and washed with PBS (pH 7.2) and eluted using 50 mM sodium citrate (pH 4.0). The EDTPA modified zirconia column was equilibrated and washed with MES buffer (20mM MES, 4 mM EDTPA, 50 mM NaCl, pH 5.5) and eluted in MES buffer containing 1 M NaCl. Samples were loaded and eluted at flow rates ranging from 0.25-0.5 ml/min. The pH of the elution was immediately increased to 7 using 500 mM Tris buffer (pH 9.0). The antibody was later concentrated and transferred into PBS by 10 fold diafiltration using a separate Pellicon XL50 ultrafiltration device. The S25 antibody was quickly frozen in liquid nitrogen at 1 mg/ml (BCA Assay) and was stored at -80°C for long-term storage.

**Bradford / BCA Assays**

The total protein content for the purified chimeric antibody and the culture supernatant were determined using either a Bradford reagent (Sigma) or BCA reagent (Pierce). BSA was used as a protein standard. For the Bradford assay, a 1 ml sample was mixed with 1 ml Bradford Reagent (Sigma). The samples were incubated for 30 min at 37°C and the absorbance at 595 nm was determined on a spectrophotometer. The BCA assay was used to determine the final concentration of the S25 antibody product. For the BCA assay, 50 μl sample or standard was mixed with 1 ml BCA reagent mixture (Pierce), containing a 1:50 dilution of reagents A and B. Human IgG and BSA were used as standards. The samples were incubated for 30 min at 37°C and the absorbance at 562 nm was determined on a spectrophotometer.

**SDS-PAGE / Western Blotting**

Samples were diluted in phosphate buffered saline (PBS) to 60 μl and 20 μl loading buffer (0.5 M Tris-HCl, 20% SDS, 40% glycerol, 10% β-mercaptoethanol, 0.1% bromophenol blue) was added. For non-reducing gels, the loading buffer lacked β-mercaptoethanol. Samples were boiled for 2 min and resolved on 10-12% Tris-glycine polyacrylamide gels (Invitrogen). The gels were run for 2-4 h at 125 V using an XCell SureLock Mini-Cell (Invitrogen) containing running buffer (50 mM Tris, 300 mM glycine, 0.1% SDS). The gels were transferred to nitrocellulose in an XCell SureLock Mini-Cell module for 6 h at 25V in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol, pH 8.3). Blots were blocked with 5% nonfat dry milk in TD buffer (140 mM NaCl, 5 mM KCl, 0.4 mM $Na_2HPO_4$, 25 mM Tris) for 2 h at room temperature. Recombinant chimeric monoclonal antibody was detected by incubating with 0.5 μg/ml goat anti-human IgG (whole molecule specific)(Sigma) in 5% nonfat dry milk in TD buffer for 1 h at room temperature. The protein bands were detected by incubating with ECL (Amersham) and exposing to film. Human IgG (Sigma) was used as a positive control.

**BIAcore Activity Assay**

The S25 antibody affinity and binding kinetics were measured by surface plasmon resonance in a BIAcore (Pharmacia Biosensor). The method for determination of antibody affinity was previously published [3]. Briefly, Purified IgG in 10 mM acetate (pH 3.5-4.5) was coupled to a CM5 sensor chip using N-hydroxysuccinimide-N-ethyl-N'-(dimethylaminopropyl)-carbodiimide chemistry. The association constant ($k_{on}$) for purified BoNT serotype A Hc was measured under continuous flow of 15 μl/min. The dissociation constant ($k_{off}$) was determined using a single chain variable fragment variable with specificity for BoNT serotype A Hc at a flowrate of 30 μl/min. The equilibrium dissociation constant ($K_d$) was calculated as $k_{off}/k_{on}$.

**RESULTS AND DISCUSSION**

**Isolation of recombinant CHO-DG44 cells with high S25 antibody production**

Nearly 200 clones were screened for antibody using an ELISA specific for the Fc portion of the heavy chain of human IgG. Seventeen of the cell lines had antibody titers greater than 0.1 μg/ml, with one having an

antibody titer greater than 2 μg/ml (Figure 2). This clone (CHO-DG44 S25 #56) had the highest expression level throughout the selection process. Nine clones showed expression levels greater than 0.5 μg/ml after three days in adherent cultures (Figure 3) using an Fc specific ELISA. Figure 3 shows the concentrations of light, heavy, and whole antibody determined from separate ELISAs for each of the nine high expressing clones. It should be noted that only an Fc specific ELISA was performed for each of clones 160, 180 and 181. Similar light and heavy chain antibody concentrations were determined for each of six clones tested. It is important to have similar expression levels of both the heavy and light chains to ensure full antibody is obtained upon purification. The cell line CHO DG44 S25 #56 had light and heavy chain concentrations greater than 2 μg/ml and was therefore used for the initial production of S25 antibody in suspension culture. Each of these nine clones was transferred to increasing levels of methotrexate for gene amplification (data not shown) and into serum-free media (CHO-S-SFM II, Invitrogen) to ensure culture stability and productivity is suspension culture.

**Production of a S25 antibody in batch culture**

CHO DG44 cells were transferred to suspension culture in CHO-S-SFM II and were grown in batch culture for the production of S25 anti-BoNT serotype A antibody. The CHO-DG44 S25 #56 cell line reached a maximum cell density of 4 x $10^6$ cells/ml after 5 days in suspension culture (Figure 4A). The viability of the cultures stayed above 90% until day 6 at which point it had dropped to 80% (Figure 4B). This was confirmed by a corresponding decrease in the oxygen uptake rate. The cells reached a maximum growth rate ($\mu_{max}$) of 0.95 $day^{-1}$, and the S25 antibody reached a final average concentration of 35 μg/ml, ranging from 21-53 μg/ml in four separate runs (Figure 5). This corresponds to an average specific antibody productivity of 3.1 pg/cell/day, which is similar to that found for other recombinant antibodies prior to gene amplification [33].

**Purification of S25 antibody using rProtein A Sepharose Fast Flow resin**

S25 antibody was purified from culture supernatant after a diafiltration step. 290 ml of supernatant was concentrated to 40 ml and was then transferred to PBS (pH 7.2) using a Pellicon XL50 ultrafiltration device. Diafiltered sample was loaded onto 2 ml rProtein A Fast Flow resin and eluted by gravity flow. The flowthrough was collected and the column was washed twice with 25 ml PBS (pH 7.2). The S25 antibody was eluted in 50 mM

sodium citrate (pH 4), followed by a second elution at pH 3. Samples were analyzed by SDS-PAGE and Western blotting (Figure 6). Faint bands can be observed for the light and heavy chains of the chimeric S25 antibody (Lanes 6 and 7), corresponding to the supernatant from the CHO DG44 S25 #56 cells and the dialyzed sample, respectively. Little antibody was lost in the flowthrough and wash step. Elution at reduced pH resulted in highly purified antibody. These results were confirmed by Western blotting using a goat anti-human IgG (whole molecule specific)(Sigma)(Figure 8B).

The purification of S25 antibody with Protein A Sepharose Fast Flow provided a 76% yield (Table 1). There was little loss of antibody during the ultrafiltration step. The S25 antibody was purified 11 fold and appears to be greater than 95% pure as determined by coomassie stained SDS-PAGE. The total protein concentrations were determined by a Bradford assay using bovine serum albumin (BSA) as a standard (Table 1).

**BIAcore activity assay**

S25 antibody activity was analyzed after purification using the rProtein A Sepharose Fast Flow resin. The equilibrium binding kinetics were determined by BIAcore to ensure that the chimeric S25 antibody was active and had improved binding kinetics in comparison to the single chain variable fragment from which it was derived. The $K_d$ of the S25 antibody was $1.96 \times 10^{-9}$ $M^{-1}$, with a $k_{on}$ of $6.03 \times 10^{5}$ $M^{-1} \bullet s^{-1}$and a $k_{off}$ of $1.18 \times 10^{-3}$ $s^{-1}$. This $K_d$ is much better than that determined for the single chain variable fragment. The previously reported $K_d$ of the single chain variable fragment was $7.30 \times 10^{-8}$ $M^{-1}$, with a $k_{on}$ of $1.10 \times 10^{4}$ $M^{-1} \bullet s^{-1}$ and a $k_{off}$ of $8.10 \times 10^{-4}$ $s^{-1}$[3]. These values are also similar to those previously determined for both the S25 antibody and other antibodies [3, 34, 35].

**Comparison of S25 antibody purified using EDTPA modified zirconia, MEP-hypercel and rProtein A Sepharose Fast Flow resins**

Culture supernatant was directly loaded onto EDTPA modified zirconia, MEP-hypercel and rProtein A Sepharose Fast Flow chromatography columns (Figure 7 A-C). The loading of S25 antibody was well below the binding capacity of the rProtein A Sepharose resin. The low maximum pressure drop (3 bar) of the rProtein A Sepharose fast flow resin limits the flowrates to 90 cm/h. Approximately 6 mg of total protein containing 1.25 mg S25 antibody was loaded onto the column in 26.4 ml culture supernatant. The S25 antibody was eluted off the

rProtein A column with 50 mM sodium citrate (pH 3.0) in 0.6 column volumes (CV) neutralized to pH 7.0 with 0.17 CV of 500 mM Tris base (Table 2). The Protein A Sepharose Fast Flow column provided a yield of 74.8% as determined by a whole antibody ELISA. The purity of the antibody was determined to be 99% based on the concentration of IgG in the elution fraction determined by an ELISA divided by the total protein concentration determined by a BCA assay.

The EDTPA modified zirconia column was loaded with approximately 50 ml solution consisting of a 1:1 dilution of CHO-DG44 culture supernatant and MES loading solution (40 mM MES, 8 mM EDTPA) at a pH of 5.5. The EDTPA modified zirconia column has a smaller particle size (40 μm), which resulted in a higher initial pressure drop. However, the EDTPA modified zirconia resin can handle pressure drops exceeding 400 bar. The S25 antibody was eluted off the EDTPA modified zirconia column by increasing the NaCl concentration to 1M in 20 mM MES buffer containing 4 mM EDTPA. The S25 antibody eluted in 2.4 CV (Figure 6B) and neutralized to pH 7.0 with 80 μl of 500 mM Tris base. The elution fraction had a S25 antibody concentration of 542 μg/ml and a protein concentration of 1312 μg/ml, which corresponds to a purity of 41.3%, a 1.9 fold increase. The yield for the EDTPA modified zirconia column was 89.4% (Table 2).

The MEP-hypercel column was loaded with approximately 50 ml supernatant from the CHO-DG44 S25 cells. The column was washed with 5 CV PBS (pH 7.2) and eluted using 50 mM sodium citrate (pH 4.0). The S25 antibody was eluted from the MEP-hypercel column in 2.7 CV (4.5 ml), similar to the EDTPA modified zirconia column. The elution fraction had an S25 antibody concentration of 319 μg/ml and a total protein concentration of 729 μg/ml, which corresponds to a purity of 43.7%, a 2.1 fold increase. The yield for the MEP-hypercel column was 74.8% which is similar to that achieved using the rProtein A column. The loss of S25 antibody appears to be due to irreversible binding onto the resin since there was little antibody in either the flowthrough or the wash fractions.

**Analysis of Purification by Western blotting**

A non-denaturing SDS-PAGE gel was run to compare purity of the EDTPA modified zirconia and MEP-hypercel purified samples to that purified using rProtein A Sepharose Fast Flow (Figure 8). Both rProtein A purified samples were very pure with no visible bands corresponding to non-IgG proteins. There are numerous bands that correspond to contaminating proteins from the CHO DG44 S25 culture supernatant purified using

EDTPA modified zirconia column. The S25 elution peak from the MEP-hypercel column is significantly broader than that obtained from the other columns and has a shoulder on the front. All three fractions contain a high level of contaminating proteins and therefore were combined. This shoulder suggests that an improvement in purity could be obtained by elution at several pH steps. Comparing the EDTPA modified zirconia and MEP-hypercel peaks it was observed that the contaminating bands in the MEP-hypercel column were different from those that occurring on the EDTPA modified zirconia column. As a result, the EDTPA modified zirconia column and MEP-hypercel column were run in series to improve the purity of S25 antibody.

**Combination of EDTPA modified zirconia and MEP-hypercel purification**

The EDTPA modified zirconia column was chosen as the first purification step since it can be operated at higher pressure drops and higher flowrates. In addition, the high antibody recovery makes it the preferred choice for an initial purification step. Neither the MEP-hypercel nor the EDTPA modified zirconia columns achieved purification efficiencies close to that achieved using the rProtein A Sepharose Fast flow column. Numerous impurities that result in large bands in the S25 antibody elute taken from the EDTPA modified zirconia column were removed in the MEP-hypercel column. As a result, the product from the EDTPA modified zirconia column was loaded onto the MEP-hypercel column. The S25 antibody from the EDTPA modified zirconia column was dialyzed into PBS (pH 7.2) using an 8000 kDa MWCO dialysis membrane (Spectra). The dialyzed sample was loaded onto the MEP-hypercel column followed by a 5 CV with PBS. The load onto the MEP-hypercel column was much less than the amount of supernatant previously loaded onto the column. The S25 antibody was eluted with 50 mM sodium citrate (pH 4.0).

The flowthrough of EDTPA modified zirconia purified S25 antibody sample loaded onto the MEP-hypercel column had an absorbance of about 70 mAU (Figure 7 D). This corresponds to protein that is being removed using the MEP-hypercel column. The S25 antibody is eluted at pH 4.0 and a peak height of 370 mAU is observed, which is much lower than observed in the other columns due to the decreased antibody load. The antibody is eluted in 2 ml volume and 0.4 ml 500 mM Tris base was immediately added to bring the pH to 7.0. The final S25 antibody concentration was 164 μg/ml and the final total protein concentration was 229 μg/ml, resulting in a final purification of 71.6%, a significant improvement to the purity obtained using the EDTPA modified zirconia column alone. The

final yield for the EDTPA modified zirconia/MEP-hypercel purification was 71.8% which is just slightly less than that obtained from a single rProtein A column.

The S25 antibody purified using the EDTPA modified zirconia / MEP-hypercel columns in series was run on reducing SDS-PAGE gel, along with the samples purified using the EDTPA modified zirconia, rProtein A Sepharose Fast Flow, and the MEP-hypercel alone (Figure 10). Comparison of lanes 6, 7, and 9 shows the improvement in S25 antibody purity obtained after running both columns in series.

## CONCLUSION

An antibody against BoNT serotype A was produced in CHO-DG44 cells and was then purified. The combination of EDTPA modified zirconia and MEP-hypercel provided an initial purification of monoclonal antibodies, but further downstream processing steps or improvements in separation conditions are needed to approach the purity achieved using a single protein A resin. While EDTPA modified zirconia does not approach Protein A resins for purity, the ability to operate at increased pressures, the high yield, and the ease of cleaning makes it an ideal capture step for the purification of a monoclonal antibodies from culture supernatant. In addition, EDTPA modified zirconia and MEP-hypercel prove to be complimentary purification steps as demonstrated by the large increase in purity obtained when running these steps in series.

## REFERENCES


[1] J. Reichert, Monoclonal antibodies in the clinic, Nat Biotechnol. 19 (2001) 819-822. Review.

[2] T. Gura, Therapeutic antibodies: magic bullets hit the target, Nature. 417 (2002) 584-6.

[3] A. Nowakowski, C. Wang, D. Powers, P. Amersdorfer, T. Smith, V. Montgomery, R. Sheridan, R. Blake, L. Smith, J. Marks, Potent neutralization of botulinum neurotoxin by recombinant oligoclonal antibody, Proc Natl Acad Sci U S A. 99 (2002) 11346-50.

[4] S. Arnon, R. Schechter, T. Inglesby, D. Henderson, J. Bartlett, M. Ascher, E. Eitzen, A. Fine, J. Hauer, M. Layton, S. Lillibridge, M. Osterholm, T. O'Toole, G. Parker, T. Perl, P. Russell, D. Swerdlow, K. Tonat, Botulinum toxin as a biological weapon: medical and public health management, JAMA. 285 (2001) 1059-70. Review.

[5] D. Lacy, R. Stevens, Sequence homology and structural analysis of the clostridial neurotoxins, J Mol Biol. 291 (1999) 1091-104.

[6] N. Mahant, P. Clouston, I. Lorentz, The current use of botulinum toxin, J Clin Neurosci. 7 (2000) 389-94. Review.

[7] L. Siegel, Human immune response to botulinum pentavalent (ABCDE) toxoid determined by a neutralization test and by an enzyme-linked immunosorbent assay, J Clin Microbiol. 26 (1988) 2351-6.

[8] M. Byrne, L. Smith, Development of vaccines for prevention of botulism, Biochimie. 82 (2000) 955-66. Review.

[9] R. Hibbs, J. Weber, A. Corwin, B. Allos, M. Abd el Rehim, S. Sharkawy, J. Sarn, K. McKee Jr., Experience with the use of an investigational F(ab')2 heptavalent botulism immune globulin of equine origin during an outbreak of type E botulism in Egypt, Clin Infect Dis. 23 (1996) 337-40.

[10] T. Gelzleichter, M. Myers, R. Menton, N. Niemuth, M. Matthews, M. Langford, Protection against botulinum toxins provided by passive immunization with botulinum human immune globulin: evaluation using an inhalation model, J Appl Toxicol. 19 (1999) Suppl 1: 35-8.

[11] G. Urlaub, L. Chasin, Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity, Proc Natl Acad Sci U S A. 77 (1980) 4216-20.

[12] F. Alt, R. Kellems, J. Bertino, R. Schimke, Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells, J Biol Chem. 253 (1978) 1357-70.

[13] R. Kaufman, P. Sharp, S. Latt, Evolution of chromosomal regions containing transfected and amplified dihydrofolate reductase sequences, Mol Cell Biol. 3 (1983) 699-711.

[14] N. Kim, S. Kim, G. Lee, Clonal variability within dihydrofolate reductase-mediated gene amplified Chinese hamster ovary cells: stability in the absence of selective pressure, Biotechnol Bioeng. 60 (1998) 679-88.

[15] T. Yoshikawa, F. Nakanishi, Y. Ogura, D. Oi, T. Omasa, Y. Katakura, M. Kishimoto, K. Suga, Amplified gene location in chromosomal DNA affected recombinant protein production and stability of amplified genes, Biotechnol Prog. 16 (2000) 710-5.

[16] G. Pendse, S. Karkare, J. Bailey, Effect of cloned gene dosage on cell growth and hepatitis B surface antigen synthesis and secretion in recombinant CHO cells, Mol. Cell. Biol. 40 (1992) 119-129.

[17] M. Page, M. Sydenham, High level expression of the humanized monoclonal antibody Campath-1H in Chinese hamster ovary cell, Bio/Technology. 9 (1991) 64-68.

[18] J. Goding, Use of staphylococcal protein A as an immunological reagent, J Immunol Methods. 20 (1978) 241-53. Review.

[19] R. Lindmark, K. Thoren-Tolling, J. Sjoquist, Binding of immunoglobulins to protein A and immunoglobulin levels in mammalian sera, J Immunol Methods. 62 (1983) 1-13. Review.

[20] K. Biedermann, M. Sabater, J. Sorensen, H. Fiedler, C. Emborg, Quantitative binding studies of a monoclonal antibody to immobilized protein-A, Bioseparation. 2 (1991) 309-14.

[21] M. Godfrey, P. Kwasowski, R. Clift, V. Marks, Assessment of the suitability of commercially available SpA affinity solid phases for the purification of murine monoclonal antibodies at process scale, J Immunol Methods. 160 (1993) 97-105.

[22] E. Bill, U. Lutz, B. Karlsson, M. Sparrman, H. Allgaier, Optimization of protein G chromatography for biopharmaceutical monoclonal antibodies, J Mol Recognit. 8 (1995) 90-4.

[23] A. Zamboni, I. Giuntini, D. Gianesello, F. Maddalena, F. Rognoni, D. Herbst, Production of mouse monoclonal antibodies using a continuous cell culture fermenter and protein G affinity chromatography, Cytotechnology. 16 (1994) 79-87.

[24] N. Abe, K. Inouye, Purification of monoclonal antibodies with light-chain heterogeneity produced by mouse hybridomas raised with NS-1 myelomas: application of hydrophobic interaction high-performance liquid chromatography, J Biochem Biophys Methods. 27 (1993) 215-27.

[25] O. Manzke, H. Tesch, V. Diehl, H. Bohlen, Single-step purification of bispecific monoclonal antibodies for immunotherapeutic use by hydrophobic interaction chromatography, J Immunol Methods. 208 (1997) 65-73.

[26] B. Nopper, F. Kohen, M. Wilchek, A thiophilic adsorbent for the one-step high-performance liquid chromatography purification of monoclonal antibodies, Anal Biochem. 180 (1989) 66-71.

[27] H. Graf, J. Rabaud, J. Egly, Ion exchange resins for the purification of monoclonal antibodies from animal cell culture, Bioseparation. 4 (1994) 7-20.

[28] A. Clausen, A. Subramanian, P. Carr, Purification of monoclonal antibodies from cell culture supernatants using a modified zirconia based cation-exchange support, J Chromatogr A. 831 (1999) 63-72.

[29] A. Subramanian, P. Carr, C. McNeff, Use of spray-dried zirconia microspheres in the separation of immunoglobulins from cell culture supernatant, J Chromatogr A. 890 (2000) 15-23.

[30] J. Nawrocki, M. Rigney, A. McCormick, P. Carr, Chemistry of zirconia and its use in chromatography, J Chromatogr A. 657 (1993) 229-82. Review.

[31] W. Schwartz, D. Judd, M. Wysocki, L. Guerrier, E. Birck-Wilson, E. Boschetti, Comparison of hydrophobic charge induction chromatography with affinity chromatography on protein A for harvest and purification of antibodies, J Chromatogr A. 908 (2001) 251-63.

[32] S. Burton, D. Harding, Hydrophobic charge induction chromatography: salt independent protein adsorption and facile elution with aqueous buffers, J Chromatogr A. 814 (1998) 71-81.

[33] J. Davies, M. Reff, Chromosome localization and gene-copy-number quantification of three random integrations in Chinese-hamster ovary cells and their amplified cell lines using fluorescence in situ hybridization, Biotechnol Appl Biochem. 33 (2001) 99-105.

[34] P. Liaw, G. Ferrell, C. Esmon, A monoclonal antibody against activated protein C allows rapid detection of activated protein C in plasma and reveals a calcium ion dependent epitope involved in factor Va inactivation, J Thromb Haemost. 1 (2003) 662-70.

[35] J. Quinn, R. O'Kennedy, Biosensor-based estimation of kinetic and equilibrium constants. Anal Biochem. 290 (2001) 36-46.

LIST OF FIGURES

Figure 1. Diagram of plasmid pS25. Plasmid contains the light (LC) and heavy chains (HC) of S25 antibody against BoNT serotype A, along with dhfr as a selectable marker.

Figure 2. Initial screening of CHO-DG44 transfectants using an Fc specific ELISA. One clone, CHO-DG44 S25 #56, had an S25 antibody content greater than 2 μg/ml.

Figure 3. Comparison of S25 antibody production in various CHO-DG44 S25 clones. Clones 3, 49, and 56 had the highest levels of expression. Clone #56 was chosen for initial production of S25 antibody. Whole IgG and kappa concentrations were not determined for clones 160, 180, and 181. The concentration is the amount of S25 antibody based on either a whole molecule, Fc specific or kappa specific ELISA.

Figure 4. Growth of CHO DG44 S25 #56 cell line in suspension culture. (A) Viable cell density, (B) Viability.

Figure 5. Figure 5. S25 antibody production of CHO DG44 S25 #56 cell line grown in CHO-S-SFM II media.

Figure 6. Analysis of samples from S25 antibody purification using Protein A Sepharose Fast Flow resin. A) Coomassie stain, B) Western blot. (1) Human IgG (10μg), (2) Human IgG (2 μg), (3) Human IgG (0.4 μg), (4) CHO-S-SFM II media, (5) CHO-DG44 supernatant, (6) CHO-DG44 S25 supernatant (7) Ultrafiltered using 10,000 kDa MWCO membrane, (8) Flowthrough, (9) Wash, (10) Elute (pH 4.0), (11) Elute (pH 3.0), (12) See Blue Protein Standard.

Figure 7. Separation of S25 antibody on various chromatography columns. (A) rProtein A Sepharose Fast Flow, (B) EDTPA modified zirconia, (C) MEP-hypercel, (D) MEP-hypercel (Loaded with elution from EDTPA modified zirconia column).

Figure 8. Comparison of S25 antibody purification using rProtein A Sepharose Fast Flow, MEP-hypercel, and EDTPA modified zirconia resins. (1) Human IgG (10μg), (2) Human IgG (2 μg), (3) Human IgG (0.4 μg), (4) CHO-S-SFM II media, (5) CHO-DG44 S25 supernatant, (6) rProtein A (ultrafiltered load), (7) rProtein A, (8) EDTPA modified zirconia, (9 – 11) MEP-hypercel fractions, (12) See Blue Protein Standard.

Figure 9. Reducing gel of S25 antibody purified using various EDTPA modified zirconia and MEP-hypercel resins. (1) CHO-S-SFM II media, (2) CHO-DG44 S25 #56 supernatant, (3) rProtein A Sepharose Fast Flow resin (dialyzed load), (4) rProtein A Sepharose Fast Flow, (5) EDTPA modified zirconia, (6) MEP-hypercel, (7) EDTPA modified zirconia #2, (8) Dialyzed sample from EDTPA modified zirconia #2, (9) EDTPA modified zirconia / MEP-hypercel, (10) See Blue Protein Standard.

Figure 1.

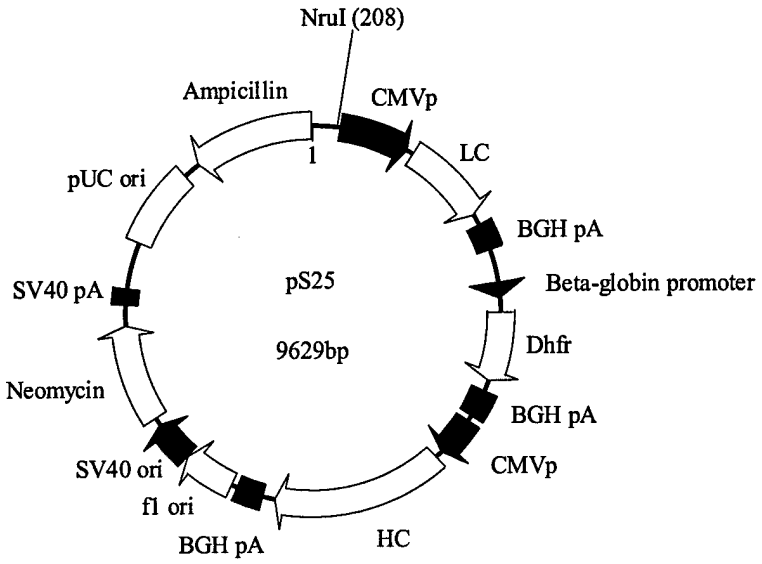

NruI (208)
Ampicillin
CMVp
1
LC
pUC ori
BGH pA
SV40 pA
pS25
Beta-globin promoter
9629bp
Dhfr
Neomycin
BGH pA
SV40 ori
CMVp
f1 ori
BGH pA
HC

Figure 2.

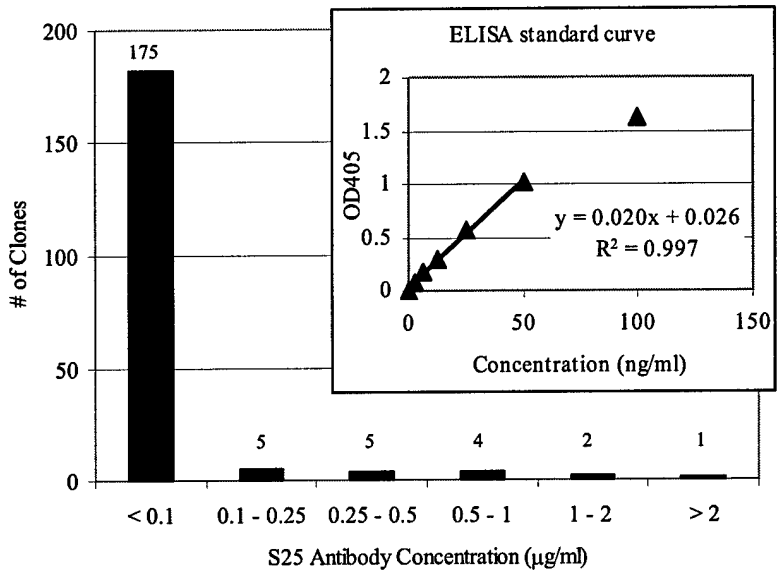

ELISA standard curve
OD405
2
1.5
1
0.5
0
0
50
100
150
Concentration (ng/ml)
y = 0.020x + 0.026
R² = 0.997
# of Clones
200
150
100
50
0
175
5
5
4
2
1
< 0.1
0.1 - 0.25
0.25 - 0.5
0.5 - 1
1 - 2
> 2
S25 Antibody Concentration (μg/ml)

Figure 3.

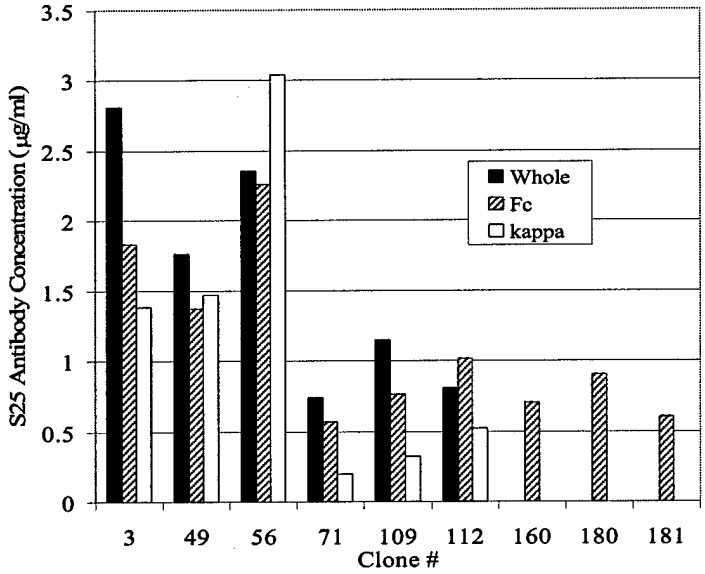

3.5
3
2.5
2
1.5
1
0.5
0
S25 Antibody Concentration (µg/ml)
Whole
Fc
kappa
3
49
56
71
109
112
160
180
181
Clone #

Figure 4.

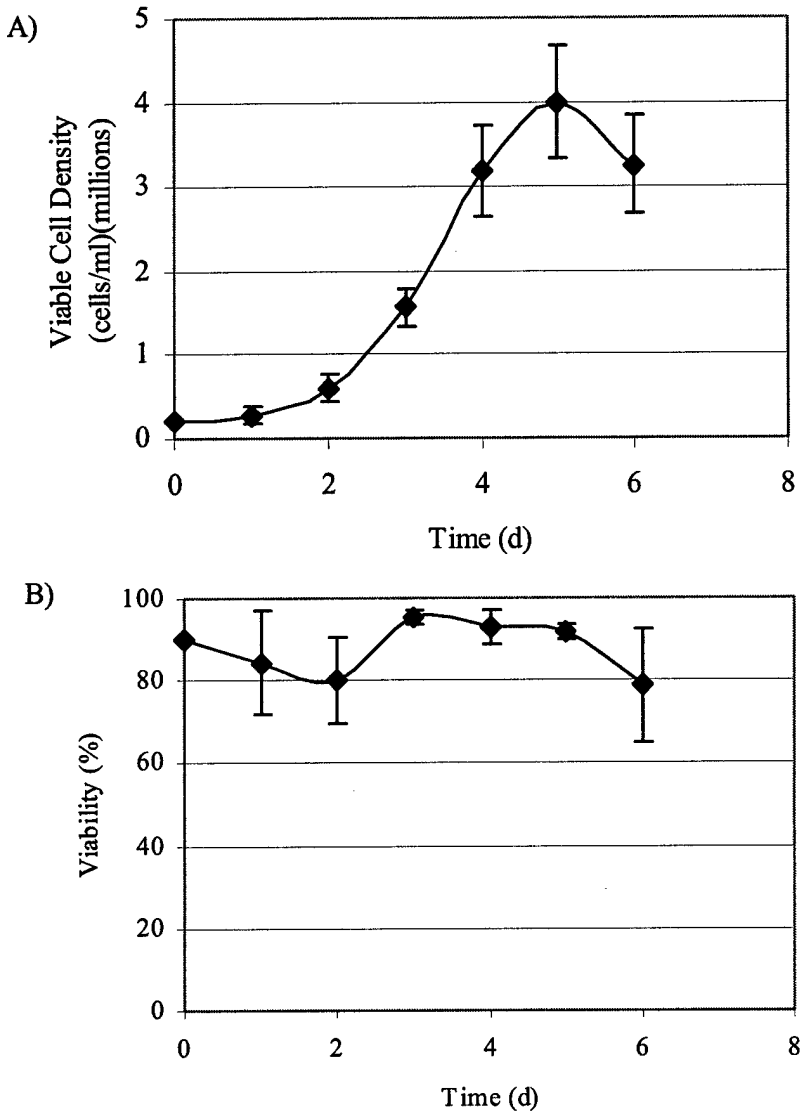

A)
Viable Cell Density
(cells/ml)(millions)
5
4
3
2
1
0
0
2
4
6
8
Time (d)
B)
Viability (%)
100
80
60
40
20
0
0
2
4
6
8
Time (d)

Figure 5.

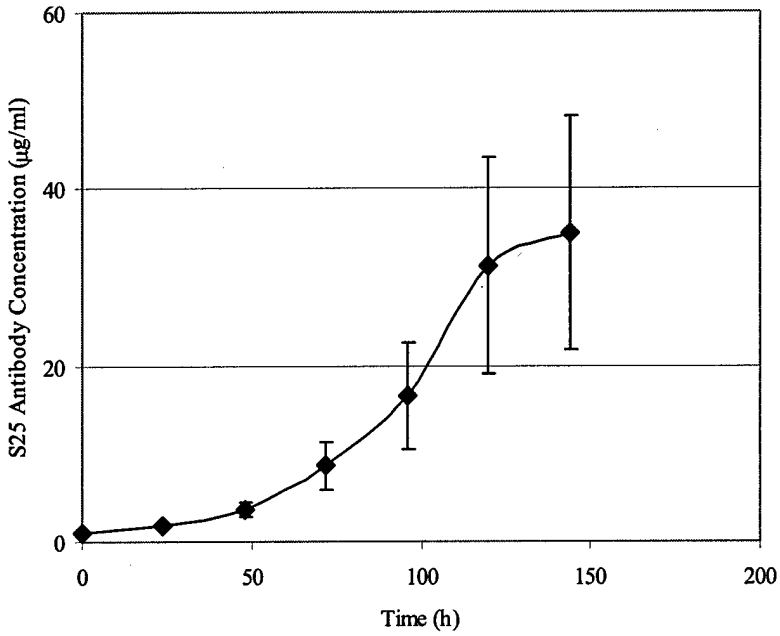
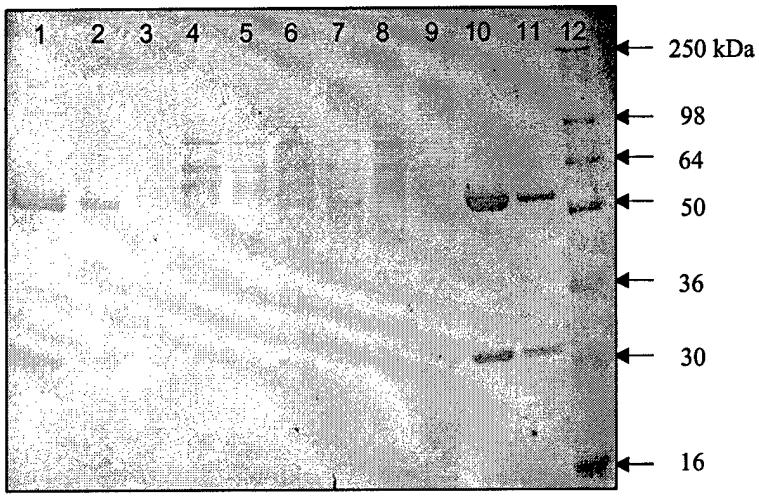

60
40
20
0
S25 Antibody Concentration (µg/ml)
0
50
100
150
200
Time (h)

Figure 6.

A)


1 2 3 4 5 6 7 8 9 10 11 12
250 kDa
98
64
50
36
30
16

B)

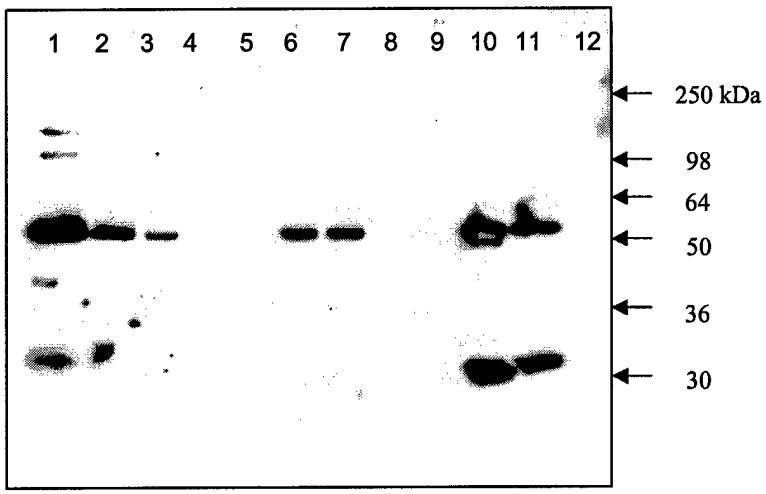

1 2 3 4 5 6 7 8 9 10 11 12
250 kDa
98
64
50
36
30

**Table 1.** Purification of S25 antibody using rProtein A Sepharose Fast Flow resin.

| | Volume (ml) | S25 IgG (μg/ml) | Total S25 IgG (mg) | Total Protein Conc. (μg/ml) | Total Protein (mg) | Yield (%) | Pruification Factor (Fold) |
|---|---|---|---|---|---|---|---|
| Supernatant | 290 | 52.5 | 15.2 | 374 | 112.2 | 100 | 1 |
| Diafiltration | 39 | 373 | 14.6 | 2689 | 104.9 | 96 | 1.03 |
| Flow Through | 39 | 3.6 | 0.14 | 1444 | 56.3 | 0 | - |
| Wash 1 | 25 | 2.6 | 0.07 | 207 | 5.2 | 0 | - |
| Wash 2 | 25 | 0 | 0 | 3.5 | 0.1 | 0 | - |
| Elution 1 (pH 4) | 27.1 | 347 | 9.4 | 238 | 6.4 | 61.8 | 10.8 |
| Elution 2 (pH 3) | 22.6 | 96.5 | 2.2 | 62 | 1.4 | 14.5 | 11.6 |
| Elute (total) | 49.7 | 233 | 11.6 | 157 | 7.8 | 75.6 | 11.0 |

Figure 7.

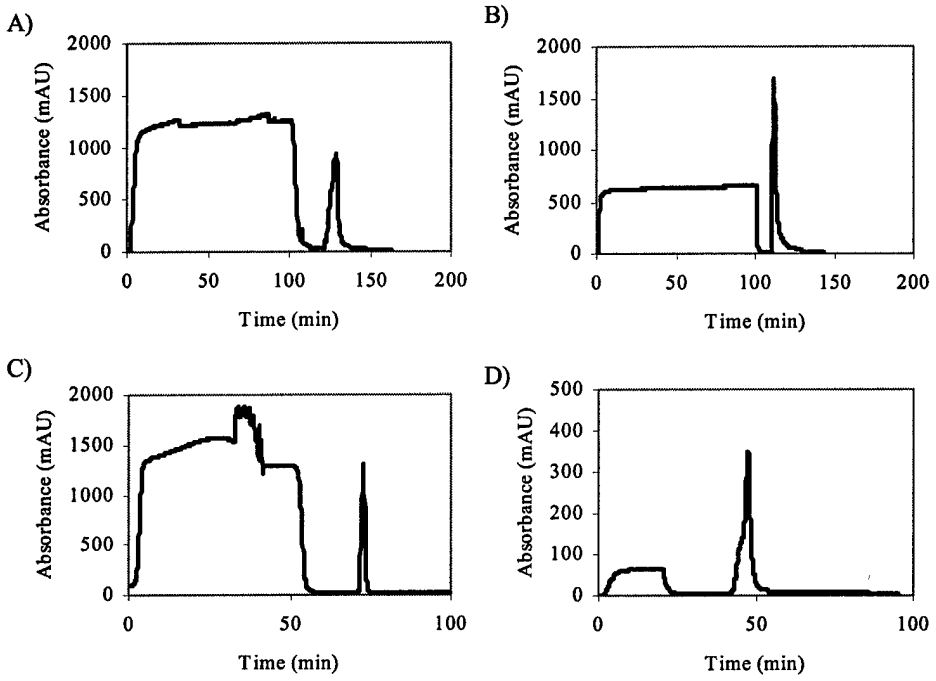

A)
B)
C)
D)
Absorbance (mAU)
Time (min)
2000
1500
1000
500
0
400
300
200
100
50
150

**Table 2.** Purification of S25 antibody using various chromatography resins.

| Sample | Volume | S25 IgG | Total S25 IgG | Protein | Total Protein | Yield | Purity |
|---|---|---|---|---|---|---|---|
| **rProtein A Sepharose** | (ml) | (μg/ml) | (mg) | (μg/ml) | (mg) | (%) | (%) |
| Supernatant | 26.4 | 47.4 | 1.25 | 228 | 6.03 | 100 | 20.8 |
| Flowthrough | 26.4 | 0 | 0 | 81.4 | 2.15 | | |
| Wash | 8.3 | 0 | 0 | 42.0 | 0.35 | | |
| *Elute | 1.28 | 731 | 0.94 | 738 | 0.95 | 74.8 | 99.0 |
| **rPEZ** | | | | | | | |
| Supernatant | 53.2 | 23.7 | 1.26 | 114 | 6.08 | | |
| Flowthrough | 53.2 | 0 | 0 | 42.1 | 2.24 | | |
| Wash | 8.3 | 0 | 0 | 20.0 | 0.17 | | |
| *Elute | 2.08 | 542 | 1.13 | 1312 | 2.73 | 89.4 | 41.3 |
| **MEP** | | | | | | | |
| Supernatant | 48.5 | 47.4 | 2.30 | 228 | 11.07 | | |
| Flowthrough | 48.5 | 0 | 0 | 53.6 | 2.60 | | |
| Wash | 8.3 | 0 | 0 | 25.3 | 0.21 | | |
| *Elute | 5.4 | 319 | 1.72 | 729 | 3.94 | 74.8 | 43.7 |
| **rPEZ/MEP** | | | | | | | |
| Diafiltration | 8.5 | 57.5 | 0.49 | 283 | 2.41 | | |
| Flowthrough | 8.5 | 0 | 0 | 12.9 | 0.11 | | |
| Wash | 8.3 | 0 | 0 | 7.8 | 0.06 | | |
| *Elute | 2.4 | 164 | 0.39 | 229 | 0.55 | 71.9 | 71.6 |

Figure 8.

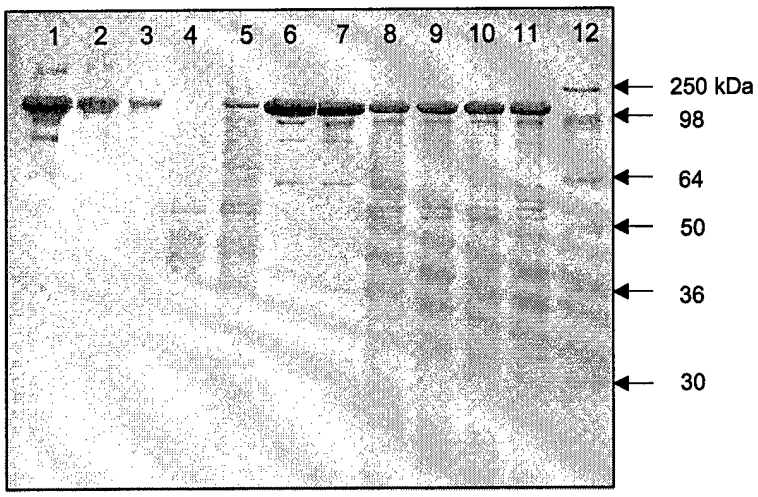
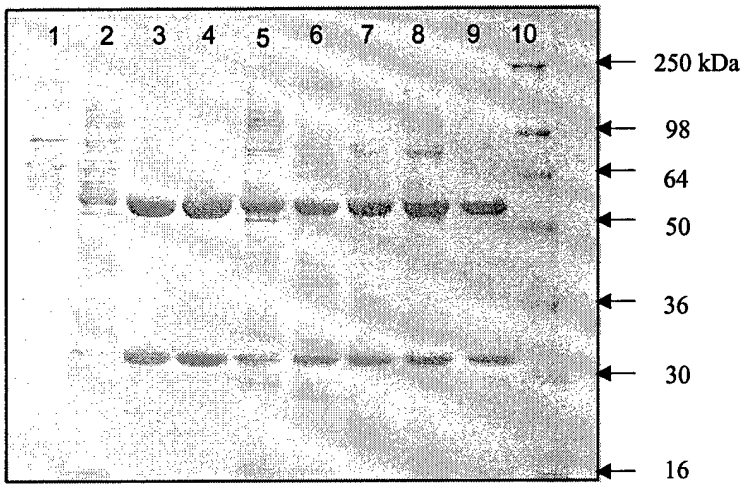


Figure 9.



**References**


1. Arnon SS, Schechter R, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Fine AD, Hauer J, Layton M, Lillibridge S, Osterholm MT, O'Toole T, Parker G, Perl TM, Russell PK, Swerdlow DL, Tonat K; Working Group on Civilian Biodefense. Botulinum toxin as a biological weapon: medical and public health management. JAMA. 2001 Feb 28;285(8):1059-70. Review.

2. Lacy DB, Stevens RC. Sequence homology and structural analysis of the clostridial neurotoxins. J Mol Biol. 1999 Sep 3;291(5):1091-104.

3. Fiock MA, Cardella MA, Gearinger NF. Studies of immunity to toxins of Clostridium botulinum . IX. Immunologic response of man to purified pentavalent ABCDE botulinum toxoid. J Immunol. 90: 697-702.

4. Siegel LS. Human immune response to botulinum pentavalent (ABCDE) toxoid determined by a neutralization test and by an enzyme-linked immunosorbent assay. J Clin Microbiol. 1988 Nov;26(11):2351-6.

5. Byrne MP, Smith TJ, Montgomery VA, Smith LA. Related Articles, Links
Purification, potency, and efficacy of the botulinum neurotoxin type A binding domain from Pichia pastoris as a recombinant vaccine candidate.
Infect Immun. 1998 Oct;66(10):4817-22.

6. Hibbs RG, Weber JT, Corwin A, Allos BM, Abd el Rehim MS, Sharkawy SE, Sarn JE, McKee KT Jr. Experience with the use of an investigational F(ab')2 heptavalent botulism immune globulin of equine origin during an outbreak of type E botulism in Egypt. Clin Infect Dis. 1996 Aug;23(2):337-40.

7. Rubin LG, Dezfulian M, Yolken RH. Serum antibody response to Clostridium botulinum toxin in infant botulism. J Clin Microbiol. 1982 Oct;16(4):770-1.

8. Nowakowski A, Wang C, Powers DB, Amersdorfer P, Smith TJ, Montgomery VA, Sheridan R, Blake R, Smith LA, Marks JD. Potent neutralization of botulinum neurotoxin by recombinant oligoclonal antibody. Proc Natl Acad Sci U S A. 2002 Aug 20;99(17):11346-50

9. Kaufman RJ, Wasley LC, Spiliotes AJ, Gossels SD, Latt SA, Larsen GR, Kay RM. Coamplification and coexpression of human tissue-type plasminogen activator and murine dihydrofolate reductase sequences in Chinese hamster ovary cells. Mol Cell Biol. 1985 Jul;5(7):1750-9.

10. Kim SJ, Kim NS, Ryu CJ, Hong HJ, Lee GM. Characterization of chimeric antibody producing CHO cells in the course of dihydrofolate reductase-mediated gene amplification and their stability in the absence of selective pressure. Biotechnol Bioeng. 1998 Apr 5;58(1):73-84.

11. Alt FW, Kellems RE, Bertino JR, Schimke RT. Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. 1978. Biotechnology. 1992;24:397-410.

12. Kim NS, Byun TH, Lee GM. Key determinants in the occurrence of clonal variation in humanized antibody expression of cho cells during dihydrofolate reductase mediated gene amplification. Biotechnol Prog. 2001 Jan-Feb;17(1):69-75.

13. Yoshikawa T, Nakanishi F, Itami S, Kameoka D, Omasa T, Katakura Y, Kishimoto M, Suga K. Evaluation of stable and highly productive gene amplified CHO cell line based on the location of amplified genes. Cytotechnology 33(1): 37-46.

14. Yoshikawa T, Nakanishi F, Ogura Y, Oi D, Omasa T, Katakura Y, Kishimoto M, Suga K. Amplified gene location in chromosomal DNA affected recombinant protein production and stability of amplified genes. Biotechnol Prog. 2000 Sep-Oct;16(5):710-5.

15. Davies J, Reff M. Chromosome localization and gene-copy-number quantification of three random integrations in Chinese-hamster ovary cells and their amplified cell lines using fluorescence in situ hybridization. Biotechnol Appl Biochem. 2001 Apr;33(Pt 2):99-105.

16. Powell KT, Weaver JC. Gel microdroplets and flow cytometry: rapid determination of antibody secretion by individual cells within a cell population. Biotechnology (N Y). 1990 Apr;8(4):333-7.

17. Manz R, Assenmacher M, Pfluger E, Miltenyi S, Radbruch A. Analysis and sorting of live cells according to secreted molecules, relocated to a cell-surface affinity matrix. Proc Natl Acad Sci U S A. 1995 Mar 14;92(6):1921-5.

18. Borth N, Zeyda M, Kunert R, Katinger H. Efficient selection of high-producing subclones during gene amplification of recombinant Chinese hamster ovary cells by flow cytometry and cell sorting. Biotechnol Bioeng. 2000-2001;71(4):266-73.

19. Heath C, Dilwith R, Belfort G. Methods for increasing monoclonal antibody production in suspension and entrapped cell cultures: biochemical and flow cytometric analysis as a function of medium serum content. J Biotechnol. 1990 Jul;15(1-2):71-89.

20. Strutzenberger K, Borth N, Kunert R, Steinfellner W, Katinger H. Changes during subclone development and ageing of human antibody-producing recombinant CHO cells. J Biotechnol. 1999 Apr 15;69(2-3):215-26.

21. Gubin AN, Reddy B, Njoroge JM, Miller JL. Long-term, stable expression of green fluorescent protein in mammalian cells. Biochem Biophys Res Commun. 1997 Jul 18;236(2):347-50.

22. Gu MB, Kern JA, Todd P, Kompala DS. Effect of amplification of dhfr and lacZ genes on growth and b-galactosidase expression in suspension cultures of recombinant CHO cells. Cytotechnology 9:237-245.

23. Fang Y, Huang CC, Kain SR, Li X. Use of coexpressed enhanced green fluorescent protein as a marker for identifying transfected cells. Methods Enzymol. 1999;302:207-12.

24. Kito M, Itami S, Fukano Y, Yamana K, Shibui T. Construction of engineered CHO strains for high-level production of recombinant proteins. Appl Microbiol Biotechnol. 2002 Dec; 60(4):442-8.

*Key Research Accomplishments*

- Construction of the shell space on third floor is nearly completed.
- Generated CHO-DG44 cell line (CHO-DG44 S25 #56) with S25 antibody production
- Manuscript on production and purification of S25 antibody from CHO-DG44 cells has been submitted.