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THE USE OF A RESIN BOUND MONOCLONAL ANTIBODY IN THE
PURIFICATION OF THE VARIOUS COMPONENTS IN ANTHRAX VACCINES

Annual/Final Report

Joseph W. Burnett, M. D.
Gary J. Calton, Ph.D.

20 August 1987

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-84-C-4146

University of Maryland School of Medicine
Department of Medicine
Division of Dermatology
Baltimore, Maryland 21201

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FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission of Life Sciences, National Research Council (NIH Publication No. 86-23, Revised 1985).



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→ The results of the research supported by this contract have been recorded in eight papers.) Three of which have been published, one accepted for publication, one resubmitted after review, one initially submitted and two are presently (September, 1987) in manuscript form. → The papers can be divided into two equal groups. The first describes the purification techniques performed to independently isolate the three exotoxins of Bacillus anthracis: protective antigen (PA), lethal factor (LF) and edema factor (EF). The sequential purification technique of the three exotoxins is described in an additional paper. These papers delineate a good technique for isolating all of the exotoxins individually using columns prepared with their own specific monoclonal antibody. An alternative method is to purify the crude fermentation bacterial broth by removing PA and LF and allowing the residue to serve as EF. This method is almost as effective as preparing EF alone from its own specific monoclonal antibody columns.)

The second group of four papers describes the methods by which immunosorbent chromatography should be designed in order to obtain the best results when isolating antibodies or exotoxins. In summary, it has been found that every monoclonal antibody will require its own specific linkage to an inert support in order to perform optimally.

→ The specific points uncovered by our research ^{are herein} will be summarized in paragraphs corresponding to the paper in which they were or will be published. ~~the main topics are:~~

I. → Studies on the specific exotoxins of Bacillus anthracis.

A. → Purification of lethal factor.

LF could be purified many fold by immunosorbent chromatography utilizing an anti-lethal factor monoclonal antibody Sepharose 4B column. The antibody was attached to the cyanogen bromide activated Sepharose column. LF bound to the column at pH 7 in 0.05 M sodium phosphate buffer and was eluted with 4 M NaSCN buffer with 77% of the immunological activity. Pre-elution of the column with 4 M NaCl was very effective in removing non-specific binding proteins. Material isolated from this column was composed of a single component on SDS gels having a molecular weight of 82,000 and was greater than 99% pure. The antigen-antibody complex on the column was found to undergo a reversible dissociation at high pH values. The ionizable group responsible for this dissociation had a pK_a value of 9.9. The purified lethal factor exhibited a significant decrease in immunological activity upon freeze-thawing, with 52% loss in potency observed after three cycles. NaSCN buffers had the disadvantage of interfering with the results of antigenicity obtained on an ELISA plate, therefore, dialysis of each fraction immediately after elution from the column was required.

B. → Protective antigen. → ~ ~ ~

PA could be removed from a crude fermentation supernatant of B. anthracis by columns prepared with cyanogen bromide activated Sepharose CL-4B resin. The immunosorbent (4 mg monoclonal antibody/mL resin) had the capacity to bind 1 mg PA/mL resin. At this level, PA was completely absorbed from the inoculum and could then be eluted with 2 M NaSCN to result in complete recovery of PA based on antigenic activity after consideration of the antigenic degradation of PA by NaSCN. The MAB column had a flow rate of 40 mL/hr and the isolation of 1 mg PA from a 50 mL culture supernatant both took less than 3 hr at 4°C. One problem was the significant degradation of the PA by proteolysis which has been well known by other investigators. These minor proteins produced by PA degradation, as shown by immunoblot, were removed by gel permeation chromatography with Sephadex to provide a homogeneous product. The PA isolated from this column had a molecular weight of 85,000. This

technique was found to be rapid and efficient in addition to being economical because the resin could be recycled more than 10 times without loss of binding capacity.

While the initial total antigenic activity of PA was known, the eluted activity was decreased by 50% due to degradation by NaSCN. One disadvantage of this immunopurification technique was the small but detectable contamination of the specific fraction product by murine antibody. The cyanogen bromide activation of the dextran support allowed leakage of the monoclonal antibody (see stability paper in group II).

C. Sequential purification of Bacillus anthracis exotoxins by immunosorbent chromatography.

Protective antigen and lethal factor were isolated from the crude culture supernatant of Bacillus anthracis by sequential immunosorbent chromatography using immobilized monoclonal antibodies against their respective toxins. PA, LF and EF were purified by 1.1, 6.3, and 2.3 fold, respectively, while their recovery was 63, 70 and 46%, respectively. All three toxins retained their biological activity after the purification process and were not contaminated with any of the other toxins. The order of the immunosorbent columns during the purification process was important since the LF monoclonal antibody cross reacted with PA. For this reason, PA was initially removed from the crude culture supernatants. LF was then removed second and the remaining fluid contained predominantly EF.

D. Purification of EF by immunosorbent chromatography.

Hybridomas producing EF have been difficult to produce in the past. Through a screening procedure, 20 hybridomas secreting IgG antibodies reactive with EF were selected. Of those 20, 13 were injected intraperitoneally into mice. One particular secondary clone with ELISA titer of over 1:64,000 was chosen for the immunoaffinity chromatography. The crude culture supernatant was inoculated onto the column and the specific fraction removed with 20 mL of 4 M sodium thiocyanate. These bound fractions were pooled, dialyzed, lyophilized and reconstituted before being checked for EF immunological activity using ELISA. These specific fractions were positive, whereas their flow through fraction counterparts were negative for EF by ELISA. At the time of the composition of this report the SDS acrylamide gel demonstrated two bands of proteins in the specific fractions. Preliminary data suggests that there will be enough material here to prepare a paper and when such a manuscript is ready, it will be forwarded to the granting agency.

II. Studies performed on immuno-chromatography techniques.

A. The stability of antibody attachment in immunosorbent chromatography. → over

Detachment of immobilized antibody from cyanogen bromide activated support matrices in an immunosorbent system was demonstrated. The system, however, was stable under slightly basic conditions. The detachment of the antibody occurred during the elution of antigen complex with that immobilized antibody. The antibody could be detached from the matrix by different elution buffers and was independent of the number of cycles used as well as the nature of the support material itself. A change in the molecular structure of the detached antibody occurred as revealed by an alteration in the UV absorption spectra of the released antibody. The antibody detachment from the support matrix occurred in more than one antigen-antibody system suggesting that this leakage phenomenon may be a widespread disadvantage of cyanogen bromide activation procedures.

Con + d

B. Effect of borohydride reduction on antibodies.

The effect of borohydride reducing agents on the monoclonal and polyclonal antibodies was variable since each antibody showed different stability characteristics. Sodium cyanoborohydride was milder toward the immunological activity than sodium borohydride. However, in the presence of a catalytic amount of metal ion (Zn^{+2} or Al^{+3}) the former cyanoborohydride compound can be as harsh as sodium borohydride. Activated hydrophobic borohydrides in immunosorbent chromatography did not have any advantages with respect to stability of the antibody. Antibodies to be used for immunosorbent purification should be evaluated individually to determine whether their structure is stable both to immobilization reagents and their conditions of immobilization prior to the linkage of the antibody to the column support.

C. Evaluation of activation methods with cellulose beads for the immunosorbent purification of immunoglobulins.

The absorption capacity of cellulose beads immunosorbent was as good as agarose beads or any other synthetic matrices such as fracto gel, HW-65, or trisacryl, GF 2000, moreover, the cellulose beads were able to provide a fast separation at low pressure. The combination of cellulose beads and dimethylsulfoxide-carbodiimide oxidation provided a route to obtain a high flow stable immunosorbent suitable for large scale purification of specific immunoglobulins.

D. Immobilization of antibody on cellulose bead derivatives.

In these investigations the efficiency of inert spacers attached to the cellulose bead matrix was investigated. Specifically, spacers containing 3-8 carbons were evaluated for their antibody binding capacity. It was found that the longer spacers permitted more non-specific absorption. Because of this fact if large spacers were used the inoculum had to be initially purified in order to obtain excellent results. Shorter spacers did not permit the specific binding of antibody to the degree of their immediate counterparts. Optimal performance of these columns was achieved with spacers containing 4-6 carbon atoms. Leakage of antibody from the matrix containing spacers was not a problem (see reference 1, group II) more than that observed in columns without spacers.

In summary, our investigations have shown that the three various exotoxins of Bacillus anthracis can be purified by affinity immunochromatography using specific mouse monoclonal antibodies. Additionally, each monoclonal antibody has to be individually tested in order to find optimal column binding techniques in order to improve the efficiency of the purification system. The next series of experiments, if this grant were to have been continued, would have been to study the more refined column chromatography procedures outlined in the group II papers for each of the three B. subtilis monoclonal antibodies. The experiments described in the papers from group I were performed on antibody columns which had been activated by cyanogen bromide reactions. These investigations were performed in this manner because the experiments described in the group I and group II papers were performed concurrently.

Papers Published

1. Machuga EJ, Calton GJ, Burnett JW: Purification of Bacillus anthracis lethal factor by immunosorbent chromatography. *Toxicon* 24:187-195, 1986.
2. Peng L, Calton GJ, Burnett JW. Stability of antibody attachment in immunosorbent chromatography. *Enzyme and Microbial Technology* 8:681-685, 1986.
3. Peng L, Calton GJ, Burnett JW. Effect of borohydride reduction on antibodies. *Applied Biochemistry and Biotechnology* 14:91-99, 1987.

Papers Accepted

Peng L, Calton GJ, Burnett JW. Evaluation of activation methods with cellulose beads for immunosorbent purification of immunoglobulins. *Applied Biochemistry and Biotechnology*.

Resubmitted after revision

Larson DK, Calton GJ, Little SF, Leppla SH, Burnett JW. Purification of Bacillus anthracis protective antigen by immunosorbent chromatography. *Applied and Environmental Microbiology*.

Papers submitted

Larson DK, Calton GJ, Little SF, Leppla SH, Burnett JW. Sequential purification of Bacillus anthracis exotoxins by immunosorbent chromatography. *Applied and Environmental Microbiology*.

Papers in manuscript

Bansal J, Calton GJ, Little SF, Leppla SH, Burnett JW. Purification of Bacillus anthracis edema factor by immunoaffinity chromatography.

Peng L, Calton GJ, Burnett JW. Purification of antibodies by cellulose derivatives.

Personnel Contributing to the Effort

Joseph W. Burnett, M. D.
Gary J. Calton, Ph.D.
Edward J. Machuga, Ph.D.
Doris Larson, Ph.D.
Lin Peng, Ph.D.
Jaya Bansal, Ph.D.

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