PURIFICATION AND CHARACTERIZATION OF *STAPHYLOCOCCUS AUREUS* SEROTYPE 8 CAPSULAR POLYSACCHARIDE. PRODUCTION OF MONOCLONAL ANTIBODIES SPECIFIC FOR TYPE 8 CAPSULAR POLYSACCHARIDE.

by

Elena Bocola-Mavar

Submitted In Partial Fulfillment of the Requirements

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Purification And Characterization of Staphylococcus aureus Serotype 8 Capsular Polysaccharide. Production of Monoclonal Antibodies Specific For Type 8 Capsular Polysaccharide.

Elena Bocola-Mavar

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ABSTRACT

Infections caused by *Staphylococcus aureus (S.* aureus) remain the number one cause of hospital-acquired infections. Immunocompromised patients, who are most often the target of these infections, are unable to form antibodies against the carbohydrate molecules. Therefore, they would benefit from a passive administration of pre-formed antibodies. The goals of this study were: 1) to obtain a pure cell wall carbohydrate of type 8 S. *aureus,* 2) to develop ELISA techniques for detecting anti-capsular antibodies from mouse serum, and 3) to produce monoclonal antibodies against type 8 CP. Enzyme digested bacterial carbohydrate preparations were separated by DEAE-Sephacel chromatography. Analysis of the fractions demonstrated the presence of six peaks that absorbed at 213 nm. Two tested positive for reducing sugar. One was eliminated because of teichoic acid contamination. Purified capsular polysaccharide and formalin-treated bacteria were used to develop ELISAs to test for monoclonal antibodies in sera. Monoclonal antibodies from mice immunized with *S.* aureus, type 8 failed to bind to type 8 capsular polysaccharide, but the experiments successfully detected cross-reactive binding to type 5 capsular polysaccharide.

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October 24, 2000

Dr. Diana Fagan
Department of Biological Sciences
UNIVERSITY

RE: Submission of Protocol # 02-01

Dear Dr. Fagan:

The Institutional Animal Care and Use Committee of Youngstown State University has reviewed the protocol you submitted (Protocol # 02-01) titled "Hybridoma Preparation," and determined it should be unconditionally approved.

This Protocol is approved for a period of three years; however, it must be Updated annually via the submission of an Update form **prior** to the expiration date of 10/25/00. You must adhere to the procedures described in your approved request. The Institutional Animal Care and Use Committee must first authorize any modification to the project.

Sincerely,

Peter J. Kasvinsky (cc)

Dr. Peter J. Kasvinsky
Dean of Graduate Studies

PJK:cc

c: Dr. Paul Peterson, Chair
Department of Biological Sciences

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CP	Capsular polysaccharide
DEAE	Diethylaminoethyl
EDTA	Ethylenediamine-tetra acetic acid
HAT	Hypoxanthine aminophterin thymidine
HCl	Hydrochloric acid
HGPRT	Hypoxanthine guanine phosphoribosyl transferase
Ig	Immunoglobulin
NMS	Normal mouse sera
OD	Optical density
PEG	Polyethylene glycol
PBS	Phosphate buffered saline
TBS	Tris-buffered saline
TMB	3,3',5,5'-tetramethylbenzidine
MHC	Major histocompatability complex

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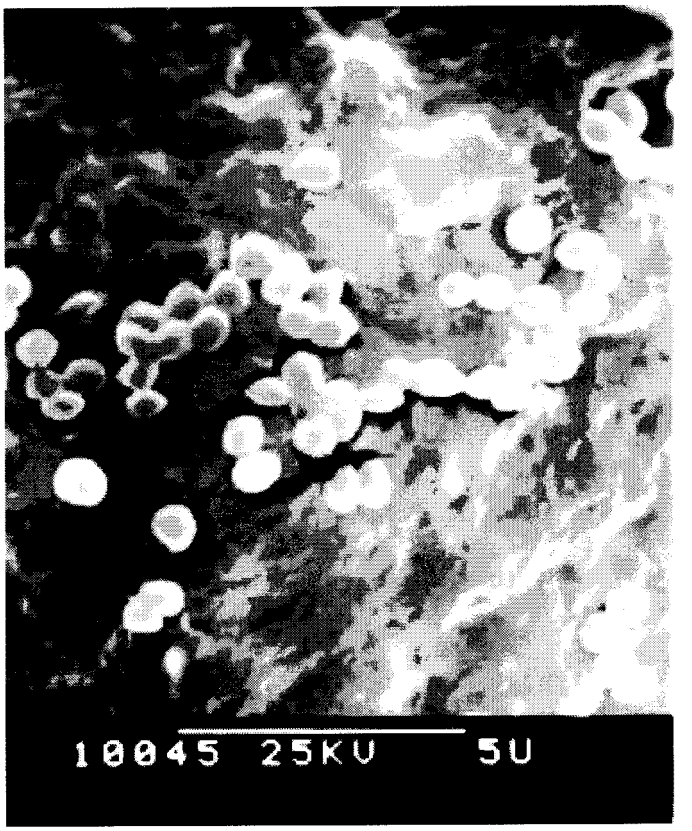
I INTRODUCTION

Staphylococcus aureus (S. aureus) is a gram-positive bacterium (Fig. 1) that causes most of the community and nosocomial (hospital-acquired) bacteremia (Tenover and Gaynes, 2000). Out of 2 million patients that acquire a nosocomial infection annually, 260,000 will be infected with S. aureus (Emory and Gaynes, 1993). S. aureus normally colonizes 20% of the population, and 30-50% of population transiently, mainly in the area of nares and perineum (Fekety, 1964). These colonizations lead to spread of bacteria to the hands of the affected individuals, where it goes on to be spread to other areas of the body, or to other people. As a result, hospitalized patients and health care workers are at a higher risk of being colonized (Fekety, 1964).

The host factors that are related to S. aureus infection depend upon the site of the infection, the patient's immunological status and the amount of the infectious dose. Skin colonization is not as effective in causing an infection as tissue or blood contamination during surgery. In addition, S. aureus is an opportunistic pathogen, so patients with compromised immunity, such as

Figure 1. ***Staphylococcus aureus*** **Appearance as Seen by Scanning Electron Microscopy.** Bacteria were cultivated in Columbia broth, supplemented with 2% Sodium Chloride. **A** sample of cells (5 ml) was taken out with a syringe and layered over a nucleopore filter. The cells were fixed with glutaraldehyde and air dried. The filter was cut to fit the mount, sputter coated and observed at 8.3 K magnification, tilt 0, aparature 3.


10045 25KV 5U

diabetics, patients on dialysis, cancer patients, HIV patients, and neonates have the highest rates of *S. aureus* infections (Frank et. al. 1997).

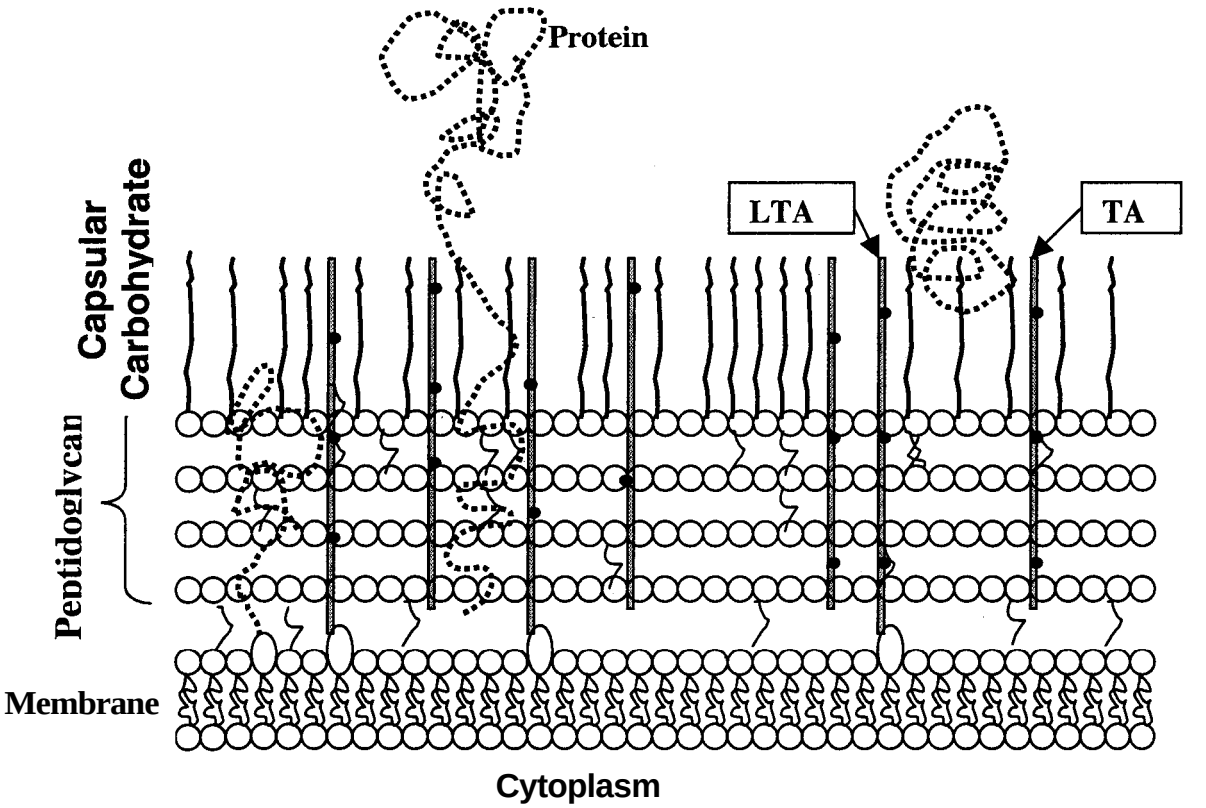
It has been repeatedly reported that nosocomial isolates of *S. aureus* have a much higher antibiotic resistance rate when compared to community isolates. Strains causing osteomylitis and bacterial endocarditis do not respond to any of the available antimicrobial treatments, thus putting patient at need for a surgical removal of the affected parts (Yu et al., 1994). The ability of *S. aureus* to mutate at high rates creates a need to develop alternate agents to treat these infections, possibly a vaccine.

In studies designed to develop a *S. aureus* vaccine, the presence of a carbohydrate capsule needs to be considered. When nosocomial strains were characterized, it was found that 90% of them have a structure covering the outer layer of their peptidoglycan, known as a capsule (Karakawa et al., 1985). Further analysis of the *S. aureus* cell wall (Fig. 2) revealed the presence of teichoic acid, which attaches to the 6-hydroxyl groups of some of the N-acetylmuramic acid residues of the glycan chain. Peptidoglycan and capsular polysaccharide surround the S. *aureus* cell in a form of a multilayer envelope.

There are 11 chemically different types of capsular polysaccharides found on the outer surface of the cell wall in S. *aureus* (Regassa, et al. 1992), which can be divided into two categories: mucoid type and microcapsule type polysaccharides. Serotypes 1 and 2 are considered mucoid type strains because they appear mucoid on solid growth medium. The remaining 9 serotypes (types 3 to 11) have microcapsules (a thin capsule layer) and are not mucoid on solid medium (Lee and Lee, 2000). The mucoid strains are rarely isolated from clinical infections (Arbeit et al., 1984). However, ninety three percent of the *S. aureus* clinical isolates and 80% of S. *aureus* isolated from sheep, goats, cows with mastitis, and chickens with osteomyelitis produce microcapsules, primarily type 5 and 8 (Fattom et al., 1996). Scientists today are focused on these two capsular types and their contribution to the virulence of S. *aureus* in general.

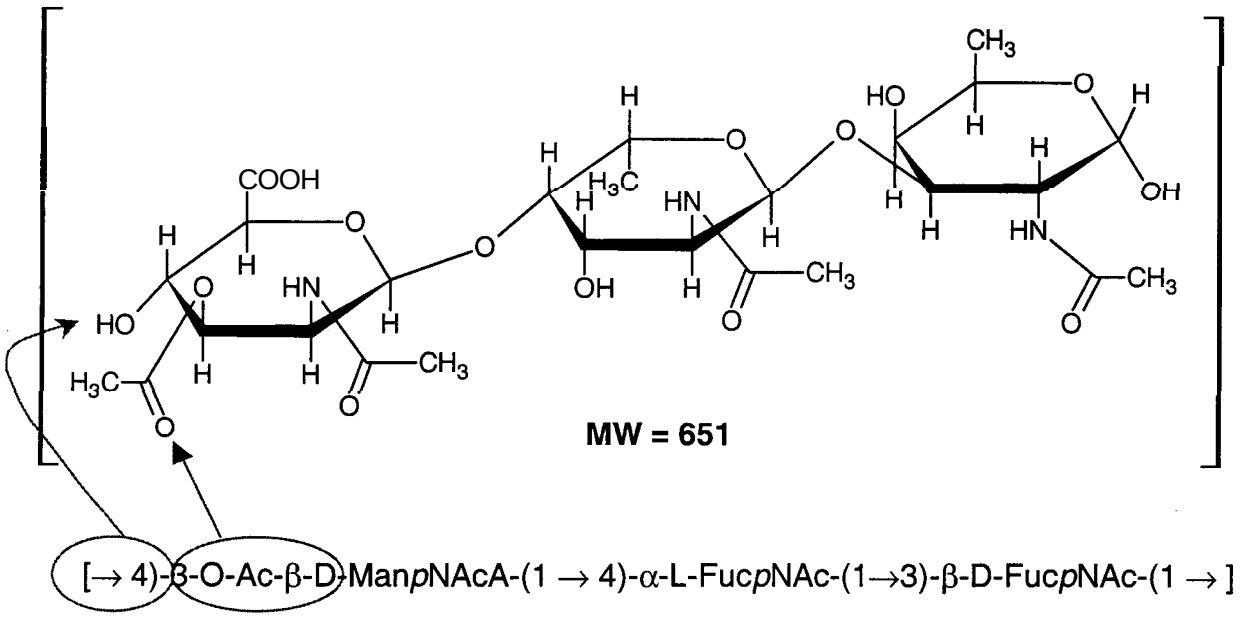
The chemical structures of type 5 and 8 capsular polysaccharides have been determined (Fig. 3). There is great similarity between the two CP structures. Furthermore, these two types have been shown to be immunologically different, with no immunological cross reactivity, or cross protection in opsonophagocytic assays (Thakker et al., 1998).

Figure 2. The Structure of the Gram-Positive Bacterial Cell Wall of *Staphylococcus aureus*. S. *aureus* is one of many gram-positive bacteria that have a layer of carbohydrate linked to their peptidoglycan layer. This is also known as capsular polysaccharide, or a capsule. Another structure associated with the capsule is teichoic acid (TA). Teichoic acid is very similar to the capsule because it is composed of a repeating carbohydrate polymer; however teichoic acid also contains phosphate. Teichoic acid is linked to the peptidoglycan layer by phosphodiester bonding. Lipoteichoic acid (LTA) is similar in structure to teichoic acid and linked to the membrane through a fatty acid (Fischetti, 2000).


Protein
LTA
TA
Capsular Carbohydrate
Peptidoglycan
Membrane
Cytoplasm

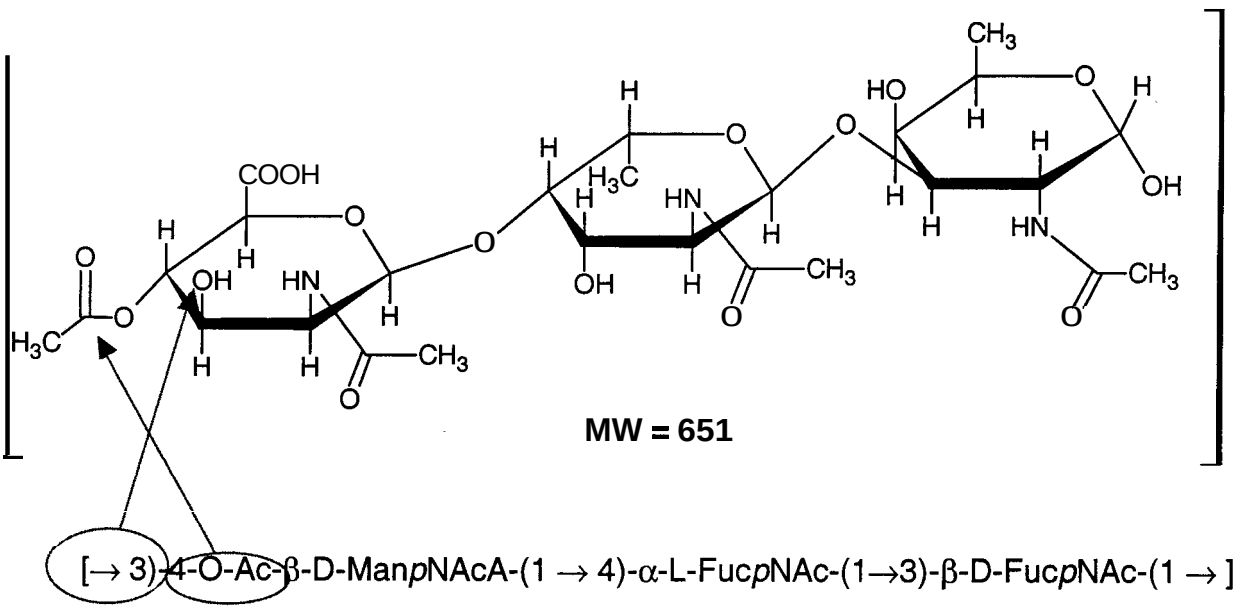
The role of microcapsules in the virulence of S. *aureus* is still not clear. Mice inoculated with encapsulated type 5 S. *aureus* had a higher frequency of arthritis and a more severe form of diseases, as compared to mice that were inoculated with noncapsulated strains (Nilsson et al., 1997). Conversely, in other studies, type 5 and type 8 microcapsule mutant strains, lacking capsular polysaccharide expression, were not less virulent than the wild type in mice injected with 50% lethal doses of live bacteria (Albus et al., 1991 and Baddour et al., 1992). However, capsular polysaccharide is believed to decrease the removal of bacteria in the host, by allowing the bacterium to resist phagocytosis and killing by polymorphonuclear leukocytes (PMNs). In order for an encapsulated bacterium to be removed, both complement and specific anticapsular antibodies are needed for its phagocytosis (ingestion and destruction by the cells of the immune system) (Verbrugh et al., 1982). Complement (a group of plasma proteins) acts as an important mediator during the immune response by amplifying the immune system's ability to destroy invading microorganisms and viruses (Goldsby et al., 2001). The effect takes place when the complement fragments bind to the complement receptors on the phagocytes, which are expressed by the invading organism. When the binding occurs, the complement activates one of its pathways to produce C3 and C5 convertases. Hydrolysis of C3 is an

Figure 3. The Structure of *Staphylococcus aureus* Serotype *5* and Serotype 8. Types 5 and type 8 microcapsules consist of trisaccharide repeating units, and share the same sugar composition that differs only in the linkage between the amino sugars and the position of O-acetylation, (Fournier et al., 1984 and Moreau et al., 1990). The repeating trisaccharide units are shown.



[→ 4)-β-O-Ac-β-D-Man*p*NAcA-(1 → 4)-α-L-Fuc*p*NAc-(1→3)-β-D-Fuc*p*NAc-(1 →]

Type 5



[→ 3)-4-O-Ac-β-D-Man*p*NAcA-(1 → 4)-α-L-Fuc*p*NAc-(1→3)-β-D-Fuc*p*NAc-(1 →]

Type 8

important amplification step which generates large amounts of C3b. C3b then binds to the bacterial surfaces, where it marks the bacterium for destruction by neutrophils and macrophages. This process is known as opsonization. S. *aureus* seems to activate and bind to complement well, but is not opsonized and phagocytosed (Wright and Silverstein, 1983). It is thought that the capsule hides the C3b, produced as a result of complement activation, which then remains unrecognized by the C3b receptors on phagocytes.

In a healthy individual, an organism entering the body is recognized as "foreign" which causes a cascade of events, the immune response, resulting in the production of antibodies and the eventual killing of the pathogen (disease causing organism). B cells that contain antibodies on their surface bind to the invader (or macrophages nonspecifically engulf any invader), ingest it and fragment it into peptides that are then expressed again on their surface to the T cells. Most importantly, activated T cells in turn activate the same B cells. B cells then proliferate to form a large number of B cell clones (B cells that have the same antibody on their surface, that is specific for that pathogen). This results in secretion of the much needed antibodies and the production of memory B cells that are going to be readily available during

the subsequent exposure to the same antigen. The pathogen is bound by antibodies that are specific for one of its surface molecules. This results in ''labeling'' of the foreign organism for destruction by the immune system. Thus the key in the immune response is the recognition of the surface molecules on the pathogen by the B cells' antibodies. The binding of antibodies then stimulates the B cell to proliferate into memory cells (long-lived) and to plasma cells, which continue to produce specific antibody until the invader is gone.

One S. *aureus* surface molecule that aids in its resistance to the host immune system is protein A. Each molecule of protein A binds strongly to two molecules of antibody via the antibody Fc region (portion of antibody that is involved in binding the complement). If the antibody binds thru its Fc region, it is not available to stimulate phagocytosis. Since are about 80,000 protein A binding sites on each bacterium, this may help to protect the bacteria from opsonization and phagocytosis (Mims et al., 2001).

The bacterial capsule also plays an important role in preventing phagocytosis of *S. aureus* bacterium. The importance of capsule in preventing the killing of type 5 *S aureus* was clear in studies where mutants,

lacking the expression of the capsule were much better recognized by macrophages, than the parental strain (Nilsson et al., 1997). The role of S. *aureus* capsule in bacterial resistance to host's defense mechanisms has made it a new target of researchers for the production of immunizations. Studies were done to see if antibodies against this capsule would protect animals from S. *aureus* infections (Albus et al., 1991, Baddour et al, 1992, Nemeth and Lee, 1994, etc). Data showed that the administration of the vaccine containing only purified type 5 and type 8 capsular polysaccharides were non-immunogenic in mice. (Fattom et al., 1990). However, when the type 5 and type 8 capsular polysaccharides were coupled to the protein carrier, antibodies were produced against the capsule that protected mice from a lethal dose of S. *aureus*. (Fattom and Naso, 1996). The reason for this is that the immune system does not respond well to the carbohydrate itself. In order to stimulate an immune response, the T cell needs to be activated, and T cell will only respond to protein molecules. For this to happen, B cells have to bind to the pathogen, break it down into the peptides (protein fragments) and present it on their surface associated with an MHC molecule. Carbohydrates will not bind to MHC molecules, and cannot be presented to T cells. T cells cannot respond to the antigen, which in turn prevents B cell from being activated by the T cell, thus poor antibody production and poor

memory formation. However, when capsule was purified from the whole bacterium, and coupled to a carrier, the carrier triggered T cell and B cell activation. This resulted in the production of antibodies against the protein carrier, along with the antibodies against the carbohydrate. It has been shown that conjugation of the CP to a protein-carrier conferred T cell dependent properties (Fattom et al., 1990). This was done by measuring the response in mice after the second injection with the conjugate. The response in these mice was much higher after the booster immunization, indicating that memory had been formed, a result of T cell activation (Fattom et al., 1990).

Immunocompromised individuals are at the highest risk for infections with S. *aureus.* The production and administration of new therapies for S. *aureus infections* is much needed for these individuals, since they are the most likely to be hospitalized. However, active immunization with the conjugate vaccine is unlikely to work in neonates, cancer, AIDS and burn patients, because they cannot form an adequate immune response. The alternate choice for these individuals would be passive immunization with hyperimmune immunoglobulin preparations (preformed antibodies). Other patients, who are otherwise healthy (shock trauma patients, cardiac surgery

patients, recipients of prosthetic devices, etc.) are likely to benefit from initial passive immunization with preformed antibodies, followed by the active immunization with conjugate, in order to induce a long lasting response (Fattom et al., 1996).

The goal of the study described will be the production of monoclonal antibodies against types 5 and 8 S. *aureus*. These antibodies would be specific for a type 5 or type 8 capsule. This would involve the purification of the cell wall carbohydrate, coupling it to the carrier and the immunization of mice. The antibody producing B cells from the mouse spleens will be isolated and fused to the myeloma cells (plasma cancer cell). The reason for this is that B cells, which are the antibody producing cells are short lived (2-3 days), so the harvested B-cells from the spleen would only produce antibodies against the immunizing agent for few days. In order to make the B-cells, thus the production of antibodies, long lived, they need to be fused to myeloma cells (cells selected to have lost the growth restrictions). This type of hybrid (hybridoma) would result in an immortal antibody-producing cell. Hybridomas will then be separated into single cells using limiting dilution (Fig. 4), and the resulting cloned cell line will produce only one type of antibody, a monoclonal antibody. These can then be tested for specificity

of binding to serotype 5 or serotype 8, using enzyme linked immunosorbent assay (ELISA).

Besides being difficult to make, the monoclonal antibodies are known to cause problems in the treatment of the disease, as they can be recognized as foreign in humans. For this reason, in future studies, the DNA from our hybridomas will be purified and DNA recombinant techniques will be used to create more potent antibodies, those that will bind tightly to the antigen and do not contain mouse antigens. These antibodies will be then used in passive imrnunotherapy, which involves directly administering pre-formed antibodies to the individuals that are not capable of forming their own antibodies, such as irnmunocompromised individuals.

II MATERIALS

Staphylococcus aureus, subspecies Rosenbach, strains: NCTC 8532 (serotype 3), Lowenstein (serotype 5), and Wright (serotype 8), P3X63-Ag8.653 (non-secreting, mouse myeloma) cells and MRC-5 (human fibroblast) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Columbia broth was from Becton Dickinson (Sparks, MD). Fetal Calf Serum (FCS) and RPMI-1640 were purchased from GIBCO BRL (Grand Island, NY). DEAE-Sephacel was purchased from Pharrnacia Fine Chemicals (Piscataway, NJ).

Tris[hydroxymethyl]aminomethane (tris buffer), Tris-ammonium chloride, monobasic potassium phosphate, hypoxanthinine-aminopterin-thymidine (HAT), magnesium sulfate, sodium bicarbonate, EDTA, toluene, ethanol, sodium acetate, sodium chloride, sodium hydroxide, lysostaphin, DNase, RNase, protease K, 2-3-5 triphenyltetrazolium chloride, liquid nitrogen, BSA, polyethylene glycol (PEG), formalin, glycerol, hydrochloric acid, n-acetyl-D-glucose, Tween-20, sulfuric acid, hydrogen peroxide, ammonium molybdate and ascorbic acid were purchased from SIGMA Chemical Company (St. Louis, Missouri). Peroxidase-conjugated goat anti-mouse Ig

was purchased from Cappel Laboratories (Cochranville, PA). BalbC mice were obtained from Jackson Laboratory (Bar Harbor, Maine).

III METHODS

A. Maintenance and Cultivation of Bacteria

1. Bacterial maintenance

The lyophilized bacterial stock was treated as instructed by the distributor. It was brought up in 1 ml nutrient broth in a sterile centrifuge tube and centrifuged at 400 *x* g for 5 minutes, at 4°C. The pellet was resuspended in a sterile 300µl glycerol and 1.7 ml nutrient broth mixture. Bacterial cells were transferred to cryogenic vials. Vials were placed in a styrofoam box, taped shut and stored at -70°C overnight. Vials were permanently stored in liquid nitrogen. Stock was maintained in 30 ml Columbia broth supplemented with 2% NaCl, as well as on nutrient agar plates, at 4°C, for up to one month. Both Gram stain and capsule stain were performed on the liquid stock.

2. Bacterial culture

Staphylococcus aureus Lowenstein (type 5) and Wright (type 8) were cultivated, as described by Fattom et al. (1990), with minor alterations. The cells were grown in Columbia broth supplemented with 2% NaCl, pH 7.5 with one liter flasks, containing between 250-300 ml of broth. Broth was inoculated with 1 ml per flask of bacterial liquid stock; and grown in a

shaking incubator at 200-250 cycles/min at 37°C, overnight. A total of six to nine liters of type 8 and type 5 were collected. The cells were harvested by centrifugation at 23,000 *x g* for 30 minutes, at 4°C and stored at -20°C until use.

B. Digestion of Bacteria

1. Enzymatic digestion of bacteria

Killed cells were digested in order to remove nucleic acids, as well as to penetrate the cell wall. This liberated the capsular polysaccharide from the other cell components. The digestion of the bacterial paste was performed according to Fattom et al. (1990). The cells were suspended at 0.5g (wet weight) per ml in 0.05 M Tris buffer supplemented with 2 mM magnesium sulfate, pH 7.5; and autoclaved at 121°C for 15 minutes. Lysostaphin (50 µg/ml) was added and incubated at 37°C overnight (with mixing). In order to assure that no viable cells were present, a sample of the solution was plated on a nutrient agar plate, and incubated at 37°C for 3 days.

Thereafter, DNAse and RNAse were added to final concentrations of 40 μg/ml each, and the incubation was continued for an additional 3 hours. The mixture was pelleted by centrifugation at 25,000 *x g* for 30 minutes, at 4°C. The supernatant was transferred to dialysis tubing to which 80 μg each of DNAse and RNAse were added per ml. The reaction mixture was dialyzed against the 0.05M Tris buffer, pH 7.5 with a drop of toluene (to help get rid off the salts from the buffer) for 3 hours at 37°C, with mixing. Protease (0.5mg/ml) was added, the outer fluid replaced with fresh buffer, and dialysis was continued at 37°C for 3 hours, with mixing.

2. Dialysis tubing preparation

Before pouring the sample into the tubing, the tubing was softened to assure that all the pores are open. Dialysis tubing had a molecular weight cut off of 13 kDa. The tubing was cut in desired lengths and prepared as described by Maniatis et al. (1982). The pieces were boiled for 10 minutes in a large volume of 2% sodium bicarbonate and 0.001 M EDTA, followed by rinsing in distilled water. The tubing was allowed to boil again for 10 minutes in a large amount of distilled water. After cooling, the tubing was stored in distilled water at 4°C until use.

C. Purification of Type 5 and Type 8 Capsular Polysaccharide (CP)

1. Precipitation of CP with ethanol

The following method was used to separate the capsular polysaccharide, the peptidoglycan layer, and the teichoic acid that it is attached to. The dialysate from the previous step was filtered through a 0.45-pm-pore-size membrane (Corning Costar, Corning, NY) and precipitated with 25% ethanol in the presence of 5mM $CaCl_2$, on ice (Fattom et al., 1990). The mixture was centrifuged at 5,000 rpm for 15 minutes, at 4°C and the supernatant was collected. Thereafter, the supernatant was precipitated with 75% ethanol, on ice, in the presence of 5mM $CaCl_2$ and the pellet was collected. The 75% ethanol precipitate was dialyzed extensively against water at 4°C , then lyophilized.

2. DEAE-Sephacel column preparation

Column preparation was performed as instructed by Pharrnacia. DEAE Sephacel solution, stored in 10% ethanol, was degassed and cleaned of fines before its application to the column. The chromatography column (Bio Rad

Laboratories, Hercules, CA) size 3 x 19.5 cm was packed with DEAE Sephacel solution. The column was washed with 2-3 column volumes of 0.05 M sodium acetate buffer, supplemented with 0.05 M NaCl, until the pH of the eluate reaches 6.0. The column was regenerated between the different serotype samples, by adding one column volume of 0.1 M sodium hydroxide. The regeneration was immediately followed with re-equilibration in starting sodium acetate buffer described above, until the eluate reaches pH of 6.0.

3. DEAE-Sephacel chromatography

The powder from step 1 was dissolved in 75 ml of 0.05 M sodium acetate-0.05 M NaCl buffer (pH 6.0) and 10 ml of the sample was stored in the refrigerator for control testing. The remaining sample was applied to the DEAE-Sephacel column equilibrated in the same buffer. The column was washed with 5 column volumes of the starting buffer, and the capsular polysaccharide was eluted with 0.15 M NaCl in the sodium acetate buffer (pH 6.0). In order to assure that CP eluted is not teichoic acid (because they both test positive by sugar testing) the carbohydrate elution was followed by teichoic acid elution with 0.2 M NaCl in 0.05 M sodium acetate buffer (pH 6.0), since it is shown previously that the teichoic acid elutes at the higher salt concentrations from the CP (Moreau et al., 1990). All of the sugar-

containing fractions were tested for the contamination with teichoic acid. Fraction collector was set to 135 drops per fraction (–7 ml).

D. Capsular Polysaccharide Characterization

1. Red Tetrazoliurn test

All of the fractions, as well as the crude sample (sample not applied to the column) were tested for the presence of reducing sugar (capsular polysaccharide) with 2,3,5-triphenyl tetrazolium chloride (Red Tetrazolium).

A small amount of the carbohydrate solution (–200 µl) was placed in a clean test tube and 1 rnl of 0.5% Red Tetrazolium aqueous solution was added, followed by one drop of 10% sodium hydroxide solution. The test tubes were placed in beaker of boiling water and the time and development of the color was noted. The absorbance of all the test tubes, as well as of the negative controls (with water) and positive controls (with N-acetyl-D-glucosamine) were read at 490 nm.

2. UV spectrophotometry

The ***UV*** absorbance at 206 nm of the fractions was taken to measure for the presence of organic compounds (Reynaud-Rondier et al., 1991). The results for absorbance at 206 nm and fractions from peaks testing positive for reducing sugars were pooled, dialyzed extensively against the Milli Q water at 4°C, and dried in the speed-vac.

3. Assay of fractions for the presence of teichoic acid phosphate

To detect the presence of teichoic acid, all the carbohydrate positive peaks were tested for the presence of phosphate, using the method described by Chen et al. (1956). **A** small amount of each sample (in lyophilized form) was dissolved in 0.5 ml of 2 M HCl, and placed in boiling water for 30 minutes. Thereafter, its volume was brought up to 4 ml with distilled water. An additional 4 ml of Reagent C (6 N sulfuric acid: distilled water: 2.5% ammonium molybdate: 10% ascorbic acid mixed together in 1:2:1:1 volumes respectively) were added to each test tube, mixed, and placed in an 37°C incubator for 2 hours. The absorbance of the test tubes was read at 820 nm on the spectrophotometer. Controls for this experiment were the following:

crude sample (sample that did not undergo the DEAE-Sephacel fractionation) before hydrolysis with HCl which liberates the

phosphate (Karakawa et al., 1972), as a negative control for phosphate.

distilled water as a negative control for phosphate.

sample of type 5 carbohydrate, provided by Pam Massullo (unpublished data) that previously tested negative for phosphate; crude sample, after the treatment with HCl, as a positive control.

- PBS, as a positive control for phosphate.

Carbohydrate samples that test negative for teichoic acid phosphate were saved and either solvolyzed and examined by NMR spectroscopy, or further separated from contaminating teichoic acid by gel exclusion chromatography.

E. Detection of Mouse Serum Antibody to Inactivated *Staphylococcus aureus* Type 3, 5 and 8 Bacteria

1. Preparation of mouse anti-type 3, 5 and 8 serum

In order to develop the method for the detection of antibody, mice were immunized with heat-killed, formalin-treated bacteria. The serum from immunized mice was used to develop ELISA for detecting of antibodies against type 5 and type 8 CP. Cultures of type 3, type 5 and type 8 bacteria

were grown in 30 ml of Columbia broth supplemented with 2% sodium chloride, for 18-22 hours at 37°C, with shaking at 225 rpm. Aliquots of each culture were tested for the number of bacterial cells remaining, by serial dilution and by plating the samples on nutrient agar plates. Formalin was added to the bacterial cultures (6 ml for every 25 ml of culture) and the cell suspension was allowed to stand at room temperature for 24 hours. The pellet was centrifuged out at 5°C for 30 minutes, at 1400 *x* g; and resuspended in 50 ml of 1X PBS (sterile filtered). The suspension was heated in a 70°C water bath, for 20 minutes and resuspended in 0.1% formalin. The samples were transferred to a sterile vaccine bottle and stored at -20°C until use. For injection of mice, the suspension was washed three times with PBS and diluted with 0.8% sodium chloride solution to a final concentration of 2 $x10^7$ cells/ml. No more than 0.5 ml were injected into a mouse.

BalbC mice were immunized by intraperitoneal injection with 0.5 ml of 10^7 cells per ml three times. The mice were than anesthetized and exsanguinated. The mouse sera were stored in aliquots at -20°C.

2. Avertin Anesthetic For Mice

The following anesthetic was used for the exsanguination by heart puncture: Avertin (25 g) was mixed with 15.5 ml of 2-methyl-2- butanol alcohol in an amber bottle and stirred on magnetic stirrer for 12 hours, or until dissolved. This made a stock solution that was kept at RT. Working solution was made fresh every 1-2 weeks by taking 0.5 ml of avertin stock solution and mixing it with 39.5 ml normal saline. This was then stirred until dissolved and filter sterilized with 0.2 micron filter. No more than 0.5 ml were administered to the mouse at one time. The mouse should be checked for the effectiveness of the anesthesia by checking the reflex response to a toe pinch.

3. Enzyme-linked immunosorbent assay (ELZSA)

ELISA was used to detect mouse sera antibody against the inactivated bacterial cells. This step was done to establish the technique for detecting the mouse sera antibody or monoclonal antibody, as needed. Types 3, 5, and 8 bacteria were cultivated overnight at 37°C in Columbia broth supplemented with 2% sodium chloride. Cells were centrifuged at 13,000 *x g* at 4°C for 30 minutes. Cell paste (10 g of each) was washed in PBS three times, and treated with 3% Formalin overnight at 4°C. It was then washed again with

PBS. Cells were then treated with trypsin (1mg/ml) at 37°C overnight, to destroy protein A (Thakker et al., 1998 and Sinha et al., 1999). The bacterial suspension was washed three times with PBS and brought up to OD_{550} of 1.0 on the spectrophotometer, according to Nelles et al. (1985). Polyvinyl microtiter plates (Becton Dickinson, Oxnard, CA 93030) and tissue culture-treated plates (Corning Glass Works, Coming, New York 14831) were coated with 100 µl of the cell suspension and left overnight at 37°C. The wells were then centrifuged at 2,000 *x g* for 15 minutes. Wells were then treated with 3% hydrogen peroxide to inactivate any endogenous peroxidases, and let stand for 15 minutes at room temperature. The wells were then washed three times in PBS. Non-specific binding of proteins was prevented by incubation of the with 1% BSA in TBS at RT for 1 hour. The wells were then coated with 1:1000 serial dilutions of mouse sera and incubated at 37°C for 2.5 hours. The plates were washed with PBS containing 0.5% Tween-20 three times. Anti-mouse peroxidase (1:1000 dilution) was added (100 µl per well) in buffer containing PBS, 0.5% Tween-20, and 0.1% BSA, pH 7.2; followed by an incubation at 37°C for 2 hours. The plates were then washed three times with PBS-0.5% Tween 20. TMB (3,3',5,5'-tetramethylbenzidine) was added to each well (100 µl per well) and the enzymatic reaction of peroxidase with TMB was stopped with

2N sulfuric acid after 10 minutes (50 µl per well). The absorbance of TMB was read at 490 nm using Bio-Tec EL311 microplate autoreader. A titer of all of the sera tested was determined (titer is a reciprocal of last dilution that tested positive for binding).

F. Production of Monoclonal Antibodies

1. Hybridoma production (cell fusion)

The spleen cells were harvested form the mice on the 3rd day after the 3rd immunization, in order to assure that the maximum number of splenocytes are producing the specific antibody against type 3, *5*, or 8 bacteria. The myeloma cell line used was P3X63-AG8.653 (ATCC). It was grown to the exponential growth phase in RPMI media containing 20% FCS. The myelomas were subcultured 24 hours prior to fusion, and approximately 2 X 10^7 cells were used for fusion with splenocytes. Hybridoma starter pack (Sigma) contained the reagent for the fusion. The feeder layer was MRC-5 adherent fibroblasts (ATCC). These were grown in EMEM media, and $2x10^4$ cells/well were used. Approximately $2x10^6$ spleen cells/ml were used. They were centrifuged at 200 *x g* for 10 minutes at RT, and 5 ml of sterile ammonium chloride was added to the pellet, followed by the addition of

Tris-buffered ammonium chloride (0.1 cells per ml of NH_4Cl) in order to lyse the red blood cells. After 2 minutes, the cells were underlayed with 2ml FCS and centrifuged at 300 x g for 10 minutes. The cells were then washed twice in RPMI+20% FCS. The fusion line in the previous step was washed twice in serum free media and centrifuged at 200 *x* g for 8 minutes. The counted spleen and myeloma cells were placed together in 50 ml conical tubes, in ration of 5 spleen cells to 1 myeloma cell. The cells were pelleted at 200 x *g* for 8 minutes and 1 ml of 50% PEG was slowly added dropwise, while shaking the tube (over the period of 1 minute). The same amount of serum free media was added for 1 minute, followed by the addition of 8 ml of serum free media over 5 minutes. The tube was then filled with the serum free media and centrifuged at 200 *x g* for 8 minutes. The pellet was resuspended in 24 ml RPMI+20% FCS and glutamine. One ml was pipetted into each well of a 24 well plate. Feeder layer (1ml) was added, together with OPI (2X total dilution) to promote the growth. The cells were maintained by incubation in a carbon dioxide supplemented incubator.

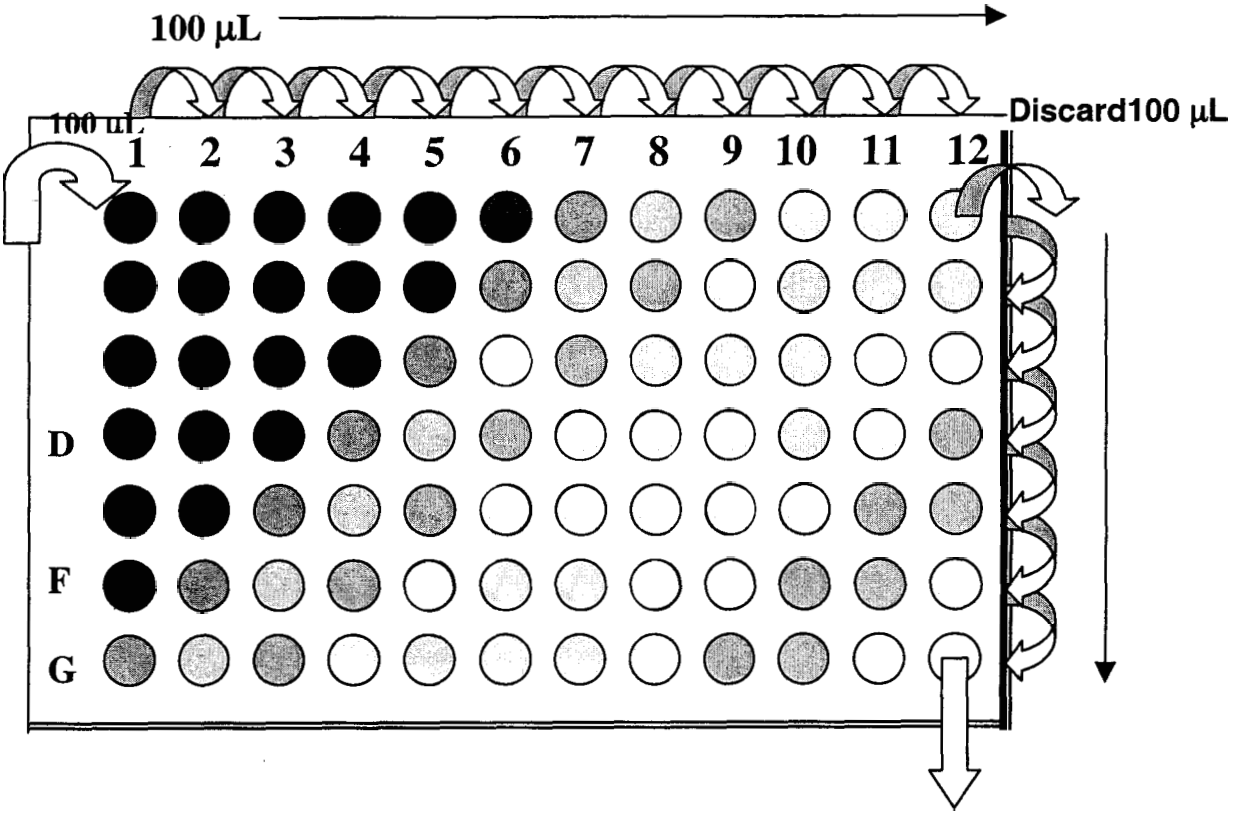
2. Selecting for monoclonal antibodies

After allowing the cells to proliferate for 1 day in the presence of supplemented RPMI media, hypoxanthine-aminoptherin-thymidine (HAT)

was added to the hybridomas in order to select for those fusions between the myeloma and the splenocyte. For two weeks, 1 ml of this media was added to each of the 24 well plates three times a week. At the end of two weeks, the cells were fed with supplemented RPMI containing HT for one week. During subsequent weeks the hybridomas were fed with supplemented RPMI. The hybridomas were then cultured in 6 well plates until there are enough cells for antibody testing by ELISA.

After the apparent growth in the well was noticed, 100 µl from the well containing hybridomas was transferred into a 96-well plate. **A** single hybridoma (monoclonal antibody) was selected for by using the limiting dilution technique (Fig. 4).

Figure 4. Limiting Dilution Technique. All the wells were added 100µl of PBS. Prepared cells (100 µl) were then placed in well A1. The cells were serially diluted by placing 100 µl of cells from well A1 to well A2, and continuing to transfer 100 µl from well to well until the last well A12. Cells (100µl) were discarded from A 12 well. The entire row A (A1-A12) was then serially diluted with a multi-channel pipette by placing 100 µl of cells from the row A, into row B, and finishing with row H. Finally, 100 µl of cells were discarded from the last row (H1-H12).


100 µL
Discard100 µL
100 µL
1 2 3 4 5 6 7 8 9 10 11 12
D
F
G
Discard 100 µl

G. Solutions

1. Supplemented RPMZ Media

The RPMI-1640 media (90ml) was supplemented with sodium bicarbonate (1.5g/1000ml), heat inactivated fetal calf serum (FCS)(20%), 29.2% L-glutamine, 0.25% glucose solution and sodium pyruvate (1ml). The pH of the solution was adjusted to 7.2-7.4. This media was used for feeding of hybridomas, as well as for maintenance of P3X myeloma cells.

2. Supplemented Earle's Minimal Essential Media (EMEM)

The Earl's MEM (90ml) was supplemented with sodium bicarbonate (1.5g/1000ml), heat inactivated FCS (10%), sodium pyruvate (1ml) and 29.2% L-glutamine. The pH of the media was adjusted to 7.3-7.4. This media was used for feeding of MRC-5 feeder cell line.

3.50% PEG

Approximately 20-50g of PEG was melted in a 100ml glass reagent bottle and melted for 20 minutes in a 55-60°C water bath. As it is cooling, but before it solidifies, an equal amount of RPMI-1640 media (unsupplemented) was added.

H. Hybridoma Cell Culture

1. Hybridoma maintenance

P3X63-Ag8.653 were maintained in RPMI-1640 supplemented media. The P3X were grown in a flat-bottomed flask in a 37°C, 5% carbon dioxide enhanced incubator. The MRC-5 were kept in a 37°C, 5% carbon dioxide enhanced incubator and maintained with supplemented Earle's minimal essential media.

IV RESULTS

Characterization of capsular polysaccharide

The isolation of the capsular polysaccharide from Staphylococcus aureus ***(S.*** aureus) bacterium was achieved by subjecting the bacterial suspension to 121°C for 20 minutes, which caused lyses of the bacterium and the liberation of capsule in the supernatant. The CP was further precipitated with 75% ethanol, and extracted from the mixture of proteins and nucleic acids using proteinase K, DNAse and RNAse. Other polymeric contaminants (teichoic acids and remaining polymeric contaminants) were removed by DEAE-Sephacel chromatography. DEAE-Sephacel fractionation was performed using sodium chloride step gradient in sodium acetate buffer. The monitoring of the DEAE-Sephacel eluate was performed at 206 nm, as described previously (Raynaud-Rondier et al., 1991). UV absorbance at 206 nm was thought to be the optimal wavelength for detecting capsular polysaccharide in the eluate. The absorbance of each fraction, shown in the overlaid sample spectra (Figs. 5 and 6), demonstrate that more of the organic material, including the carbohydrate, was absorbing at 213 nm (indicated by line through graph), rather than at 206 nm. The spectrophotometer also detected a prominent peak at 260 nm (Figs. 5 and 6), which was disregarded,

Figure 5. Overlaid Sample Absorption Spectra of Fractions Eluted With 0.05 M Sodium Acetate Buffer, Containing 0.15 M Sodium Chloride (pH 6.0). Lyophilized capsular polysaccharide (349 mg), from nine liters of *S.* aureus was dissolved in 75 ml of 0.05 M sodium acetate buffer containing 0.05 M NaCl (pH 6.0) The sample was applied to a DEAE-Sephacel column (3 by 19.5 cm; flow rate 135 drops per fraction) equilibrated in the same buffer. The unbound material was eluted with the sodium acetate buffer containing 0.05 M NaCl (equilibrating buffer). The bound carbohydrate was eluted with 0.05 M sodium acetate buffer containing 0.15 M NaCl (pH 6.0). The absorbance of individual fractions was taken on the UV/VIS spectrophotometer (Hewlett Packard, HP8452a), using wavelength range from 180 nm to 340 nm. Each line represents the absorbance of a single fraction at the specified wavelengths. Absorbance value of individual fractions is represented by the y-axis, where the different wavelengths are represented by the x-axis.

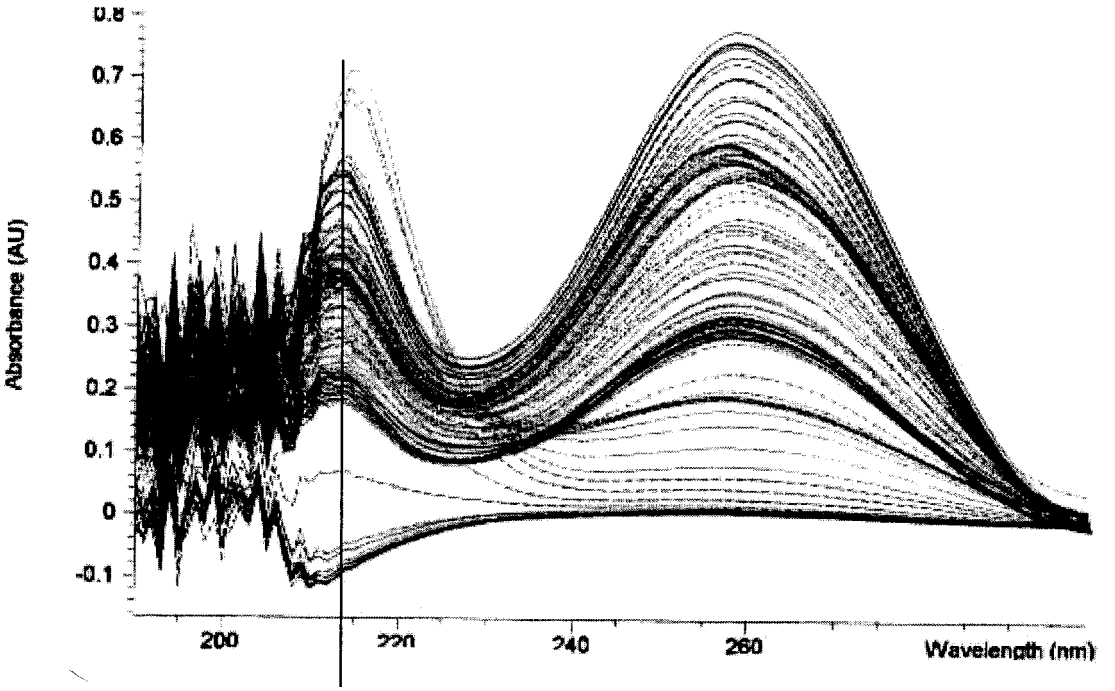
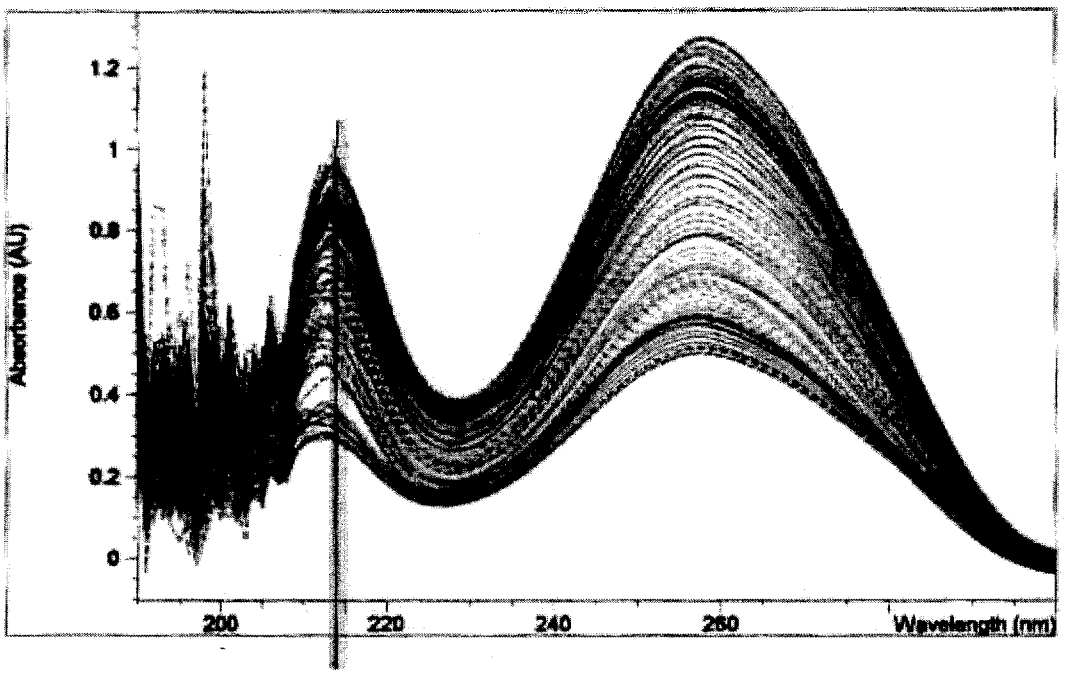

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
Absorbance (AU)
200
220
240
260
Wavelength (nm)

Figure 6. Overlaid Sample Absorption Spectra of the Fractions Eluted With 0.05 M Sodium Acetate Buffer, Containing 0.20 M Sodium Chloride (pH 6.0). The preparation of the extract used for this experiment is described in Figure 5. The absorbance of individual fractions was taken using wavelength range from 180 nm to 340 nm. Each line represents the absorbance of a single fraction at the specified wavelengths. Absorbance value of individual fractions is represented by the y-axis, where the different wavelengths are represented by the x-axis.


Absorbance (AU)
1.2
1
0.8
0.6
0.4
0.2
0
200
220
240
260
Wavelength (nm)

as this absorbance is used for the detection of nucleic acids, not polysaccharides (Fournier et al., 1984).

The sodium acetate buffer containing 0.15 M Sodium chloride was shown previously to preferentially elute carbohydrates (Fattom et al., 1990). Fractions eluted with this buffer showed three peaks at 213 nm (Fig. 7). Further elution was performed with even higher sodium chloride gradient (0.2 M), which was used previously to purify the teichoic acid (Fattom et al., 1990 and Fournier et al., 1984). It was used in this study to confirm that all of the capsular polysaccharide was eluted with the buffer containing 0.15 M sodium chloride, as well as to prove that our carbohydrate is not a carbohydrate-phosphate polymer (teichoic acid). The fractions eluted with this 0.2 M sodium chloride buffer also showed better absorbance at 213 nm and the presence of one, very broad peak (Fig. 8).

Testing for the reducing sugars (Red Tetrazolium testing)

The crude sample tested positive for the presence of the reducing sugar, using the Red Tetrazolium test developed previously (data not shown) (Fieser and Williamson (1987). Following DEAE-Sephacel chromatography, all of the eluate was tested for the presence of the reducing sugars. Fractions

Figure 7. Ion Exchange Chromatography Fraction Peaks of Type 8 Extract Eluted With 0.05 M Sodium Acetate Buffer, Containing 0.15 M Sodium Chloride (pH 6.0). Comparison Between 206 nm and 213 nm Absorbance. The extract was prepared as described in Fig. 5. The fractions and the crude sample-sample before DEAE-Sephacel fractination (data not shown) were first tested for the presence of organic compounds on the spectrophotometer by absorbance at 206 nm. After the examination of the Overlaid Sample Spectra (Fig. 5), the fractions were retested using 213 nm absorbance. Each line represents fractions eluted with sodium acetate buffer containing 0.15 M NaCl, tested by the absorbance at 206 nm (■) and 213 nm (♦). The fraction numbers are represented on the x-axis, and the individual absorbance value of each fraction is represented on the y-axis.

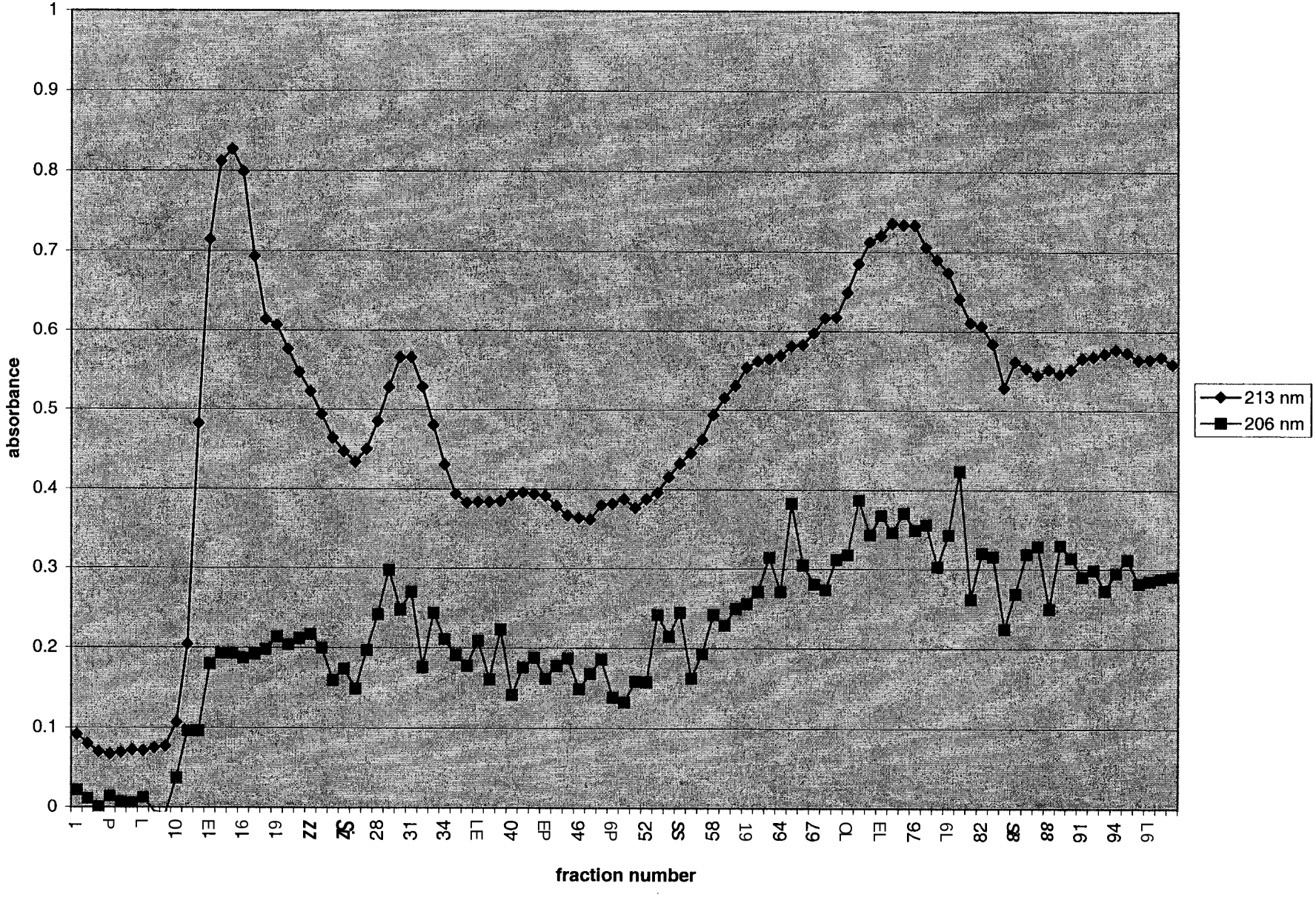
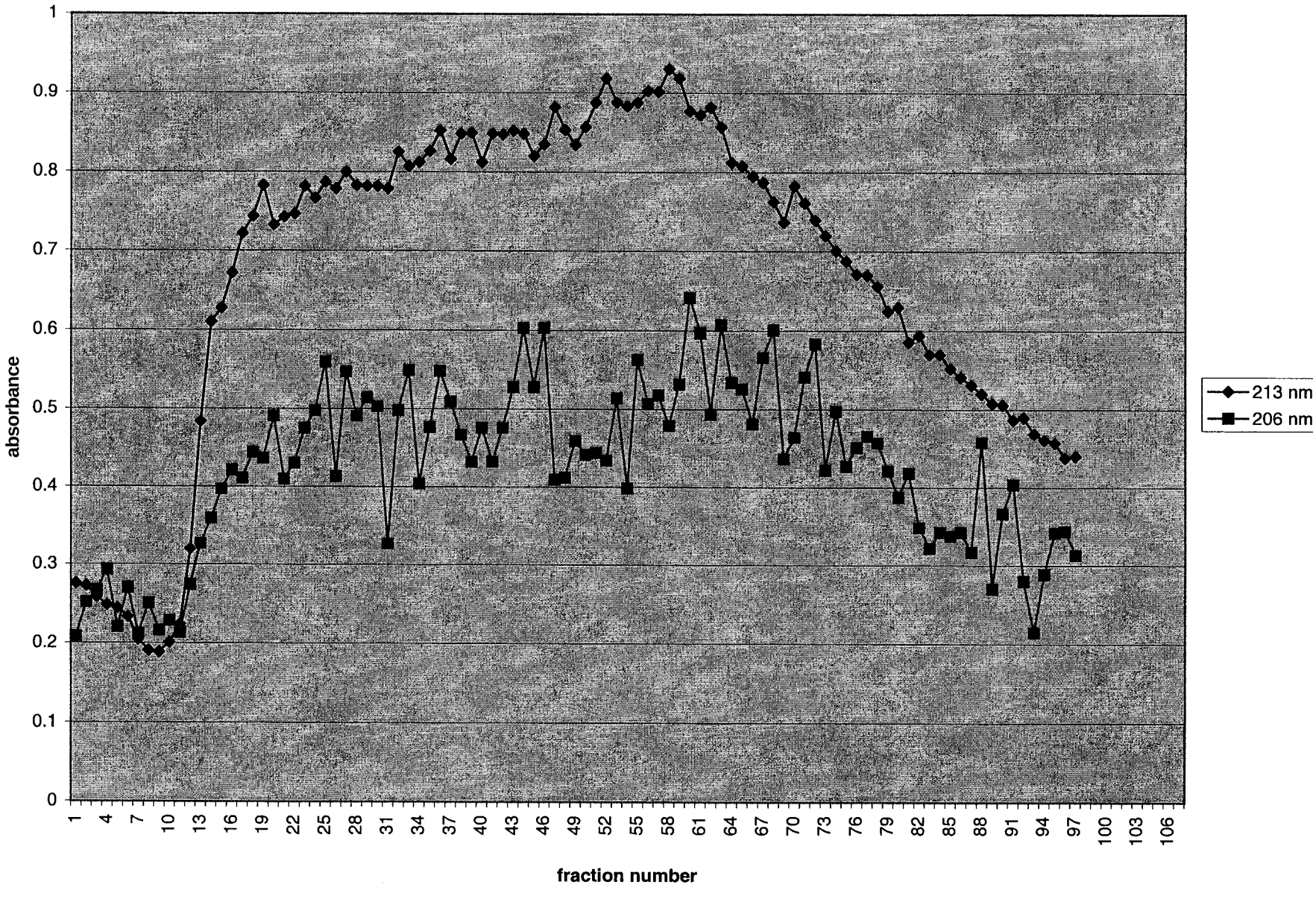

absorbance
fraction number
213 nm
206 nm

Figure 8. Ion Exchange Chromatography of Type 8 Extract Eluted With 0.05 M Sodium Acetate Buffer Containing 0.20 M Sodium Chloride (pH 6.0). Comparison Between 206 nm vs. 213 nm Absorbance. The extract was prepared as described in Figure 5. Following the elution of the carbohydrate, the teichoic acid was eluted with 0.05 M sodium acetate buffer containing 0.20 M NaCl (pH 6.0). Absorbance was taken first at 206 but after the examination of the overlaid sample spectra (Fig. 6) the absorbance was changed to 213 nm. Each line represents fractions eluted with sodium acetate buffer containing 0.20 M NaCl, tested by the absorbance at 206 nm (■) and 213 nm (♦). The fraction numbers are represented on the x-axis, and the individual absorbance value of each fraction is represented on the y-axis.


absorbance
fraction number
213 nm
206 nm

eluted with the buffer containing 0.15 M sodium chloride, showed positive reading with tetrazolium red, which peaked at the same places as the 213 nm spectra (Fig. 9, peaks # 2, 3 and 5). Red Tetrazolium also indicated the presence of sugars in fractions that were not found in the 213 nm absorbance peaks (Fig. 9, peak # 4). Since UV absorbance indicated fractions high in organic material, selecting fractions that had low UV absorbance would result in poor yield. The fractions having low absorbance at 213 nm were omitted from the further testing. The largest peak (213 nm) was seen at the beginning of the elution with the buffer containing 0.15 M sodium chloride (Fig. 9, peak # 1). Red Tetrazolium test, however, detected no reducing sugars in that peak (absorbance value of 0.05). The presence of this large peak, at the beginning of elution, was consistent with the studies performed by other laboratories (Raynaud-Rondier et al., 1991), which found that the peak consisted of primarily protein. Peaks # 2, 3 and 5, from the same buffer, were saved and had absorbances at 213 nm of 0.098, 0.117 and 0.090 respectively.

The fractions eluted with the buffer containing 0.20 M sodium chloride also contained high levels of reducing sugars (Fig. 10). In subsequent testing, these fractions were pooled together with fractions from the peak # 5 (Fig.

Figure 9. Comparison of the Red Tetrazolium Test Results and the UV Absorbance (213 nm) Results for the Fractions Eluted With 0.05 M Sodium Acetate Buffer, Containing 0.15 M Sodium Chloride (pH 6.0). The peaks from Figure 7 were further tested for the presence of reducing sugars. An aliquot (200 μl) from each fraction was tested using Red Tetrazolium test. The absorbance at 490 nm was read using microplate reader. The alignment of peaks between the fractions tested with the absorbance at 213 nm (represented by the line), and Red Tetrazolium test (represented by the column) is shown. Individual fractions for both tests are represented on the x-axis, and the absorbance values on the y-axis.

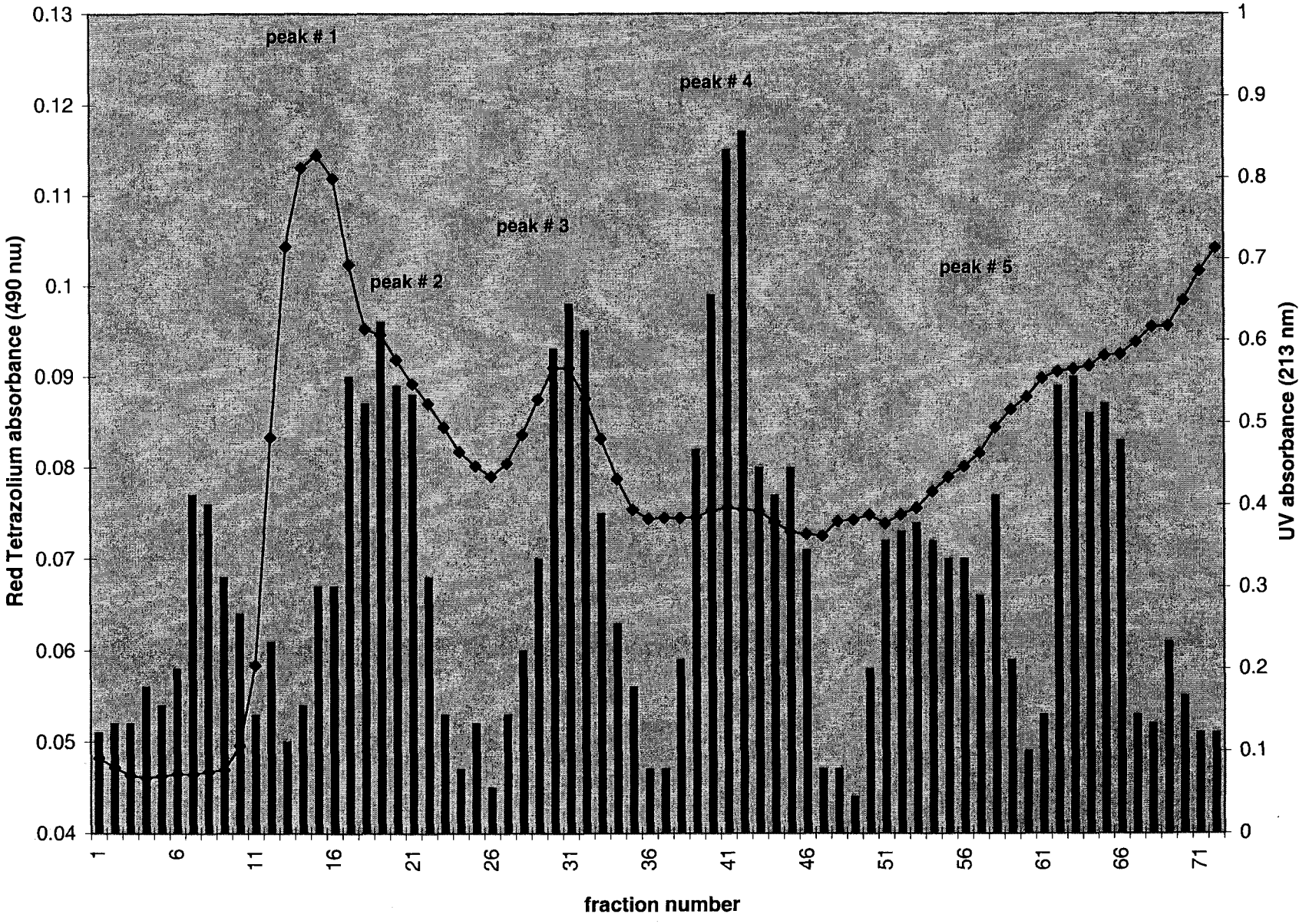
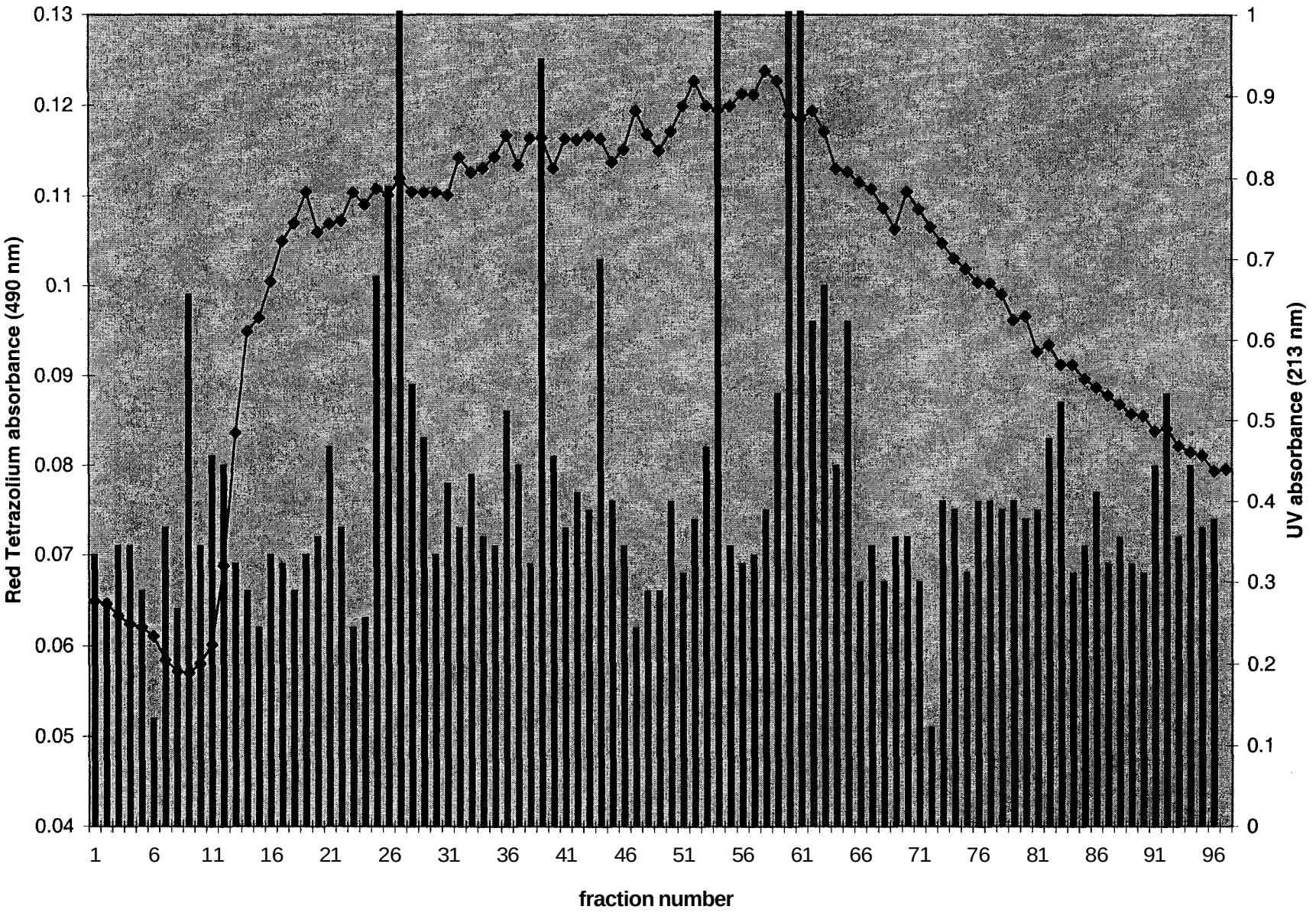

peak # 1
peak # 2
peak # 3
peak # 4
peak # 5
Red Tetrazolium absorbance (490 nw)
UV absorbance (213 nm)
fraction number
0.13
0.12
0.11
0.1
0.09
0.08
0.07
0.06
0.05
0.04
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
6
11
16
21
26
31
36
41
46
51
56
61
66
71

Figure 10. Comparison of the Red Tetrazolium Test Results and the UV Absorbance (213 nm) Results For the Fractions Eluted With 0.05 M Sodium Acetate Buffer, Containing 0.20 M Sodium Chloride (pH 6.0). The peak from Figure 8 was further tested for the presence of reducing sugars. Individual fractions were tested with Red Tetrazolium as described in Figure 9. The line represents the absorbance, and the Red Tetrazolium test results are represented by the column graph. Individual fractions for both tests are represented on the x-axis, and the absorbance values on the y-axis.


Red Tetrazolium absorbance (490 nm)
UV absorbance (213 nm)
fraction number
0.13
0.12
0.11
0.1
0.09
0.08
0.07
0.06
0.05
0.04
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
6
11
16
21
26
31
36
41
46
51
56
61
66
71
76
81
86
91
96

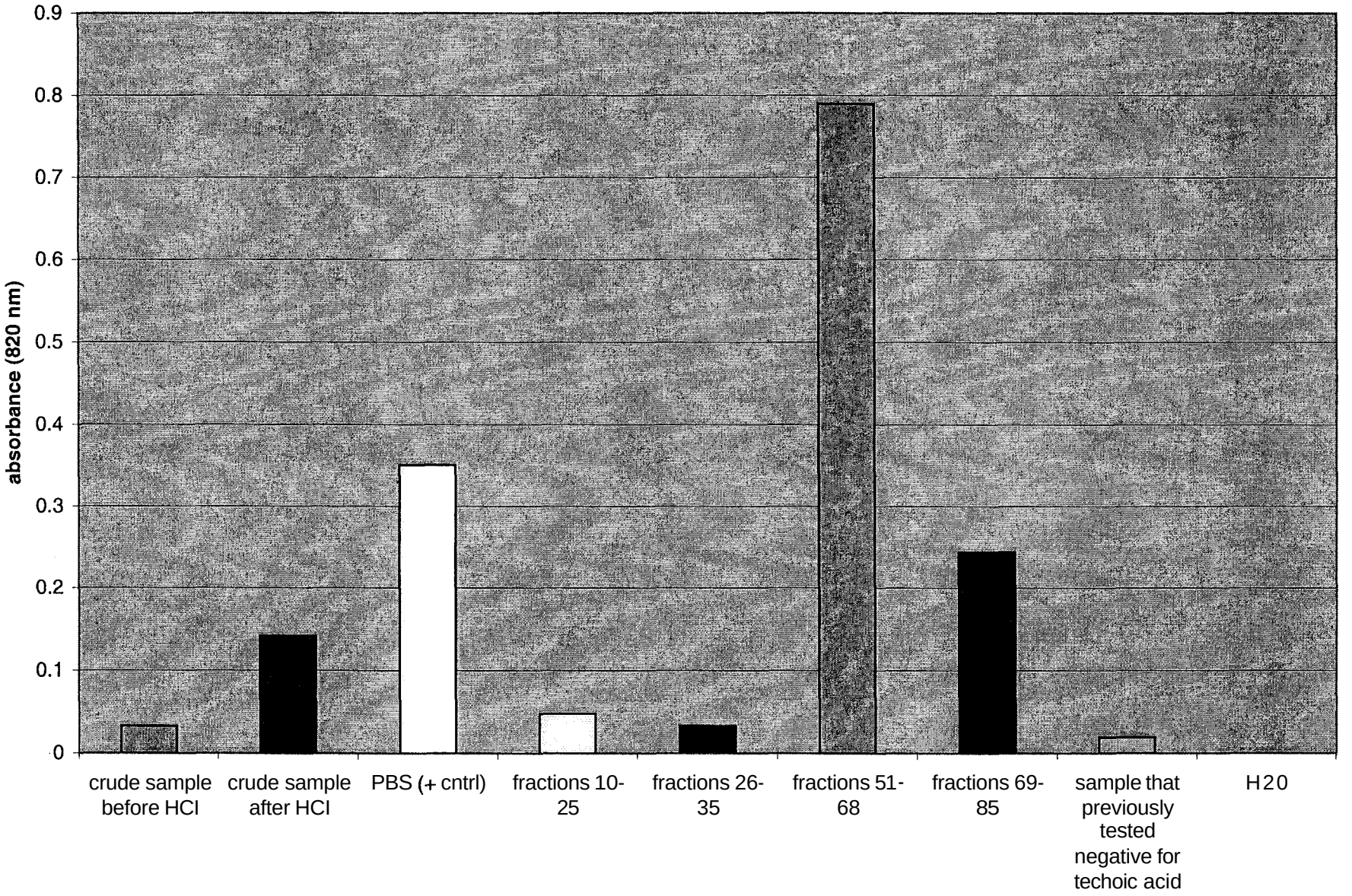
9), because it was apparent that the same molecules were starting to elute at the end of 0.15 M NaCl buffer, and continue their elution with 0.2 M buffer. This was also consistent with previous studies, which indicated that teichoic acid was eluting with 0.2 M NaCl buffer (Fournier et al., 1984).

Detection of teichoic acid contamination

S. aureus capsule preparations are usually contaminated with a carbohydrate-phosphate polymer, known as teichoic acid. The Red Tetrazolium test does not distinguish between the two, as it only tests for the presence of reducing sugars (carbohydrate). Thus, a phosphate test was performed (Fig. 11), as described previously (Chen et al., 1956), on the peaks that tested positive for sugars with the Red Tetrazolium test (Fig. 9, peaks # 2, 3 and 5). The fractions from these peaks were pooled and lyophilized. **A** small amount of each sample was tested using the Phosphate test. The change of color was read on the spectrophotometer at 820 nm.

Peaks 3 and 5 tested positive for the presence of phosphate (absorbance values of 0.79 and 0.24 respectively), indicating contamination with teichoic acid. Peak # 2 show no presence of phosphate (absorbance value 0.03), and was determined to contain the carbohydrate. Crude sample before HCl

Figure 11. Distinguishing Capsular Polysaccharide From Teichoic Acid by Testing For the Presence of Phosphate. The reducing-sugar positive peaks, containing elute of the buffer containing 0.15 M NaCl (Fig. 9), were tested for the presence of phosphate. Controls were distilled water, crude sample (sample before fractination with DEAE-Sephacel column) before hydrolysis with HCl, and purified type 5 CP. Positive controls were PBS and the crude sample after the treatment with HCl. The individual samples are represented as columns, and the absorbance values are represented on the y-axis.


absorbance (820 nm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
crude sample before HCl
crude sample after HCl
PBS (+ cntrl)
fractions 10-25
fractions 26-35
fractions 51-68
fractions 69-85
sample that previously tested negative for techoic acid
H20

hydrolysis, type 5 CP and water tested negative for phosphate. PBS gave a high reading, as expected, as well as the crude sample (before DEAE-Sephacel fractionation, and after treatment with HCl). Purified capsular polysaccharide from peak # 2 will be subjected to further analysis by NMR spectroscopy.

Binding of mouse serum antibodies to the S. aureus serotype 8 and serotype 5 whole-cell bacteria

In order to produce monoclonal antibodies against the purified carbohydrate, enzyme-linked immunosorbent assay techniques were developed to measure antibodies binding to capsular polysaccharide Preceding the availability of purified capsular polysaccharide, immunizations of mice and development of ELISA's were begun using formalin-treated bacteria. Mice were challenged i.p. with 10^7 cells/ml of the heat killed, formalin treated S. *aureus* bacteria of type 3, type 5, and type 8 (as described in Methods). Plates were coated with the heat-killed, formalin-treated type 8 bacteria.

Sera from immunized mice were serially diluted across the 96-well plate. ELISA analysis of antibody binding to S. *aureus*, type 8 showed no obvious difference between the four sera, and no decrease in binding with serial

dilutions (Figs. 12 and 13). There was, however, a slightly higher binding in non-tissue culture treated plate (Fig. 12), which was then used for all subsequent ELISAs.

Since there was no decrease in binding with the serial dilution of antibody, it was suspected that non-specific binding was occurring with the secondary antibody used. The binding of primary antibody was excluded as a possibility for error, because the decrease in binding would have been seen as the amount of this antibody was serially diluted across the plate. Since S. ***aureus*** cell wall is covered with protein A (Mims et al., 2001), it was concluded that most of the binding was coming from the anti-mouse (secondary) antibody used in ELISA binding to protein **A.** In order to improve the specificity of binding of sera to the whole cell type 8 bacteria, the preparations of cells used to coat the 96-well plate, were further treated with 1 mg/ml of trypsin in order to remove protein A (Thakker et al., 1998 and Sinha et al., 1999) (Fig. 14). Better results were obtained with the treatment of the cells with trypsin: less binding occurred with the increase in dilution of sera. However, absorbance for all sera was very erratic suggesting some technical problems still to be resolved.

Figure 12. Binding of the Mouse Immunoglobulin to Staphylococcus *aureus* Type 8 Heat-Killed, Formalin-Fixed Bacterium; Assay Performed Using Polyvinyl Chloride, Non-Tissue Culture Treated Plate.

The bacteria were cultivated as described in Methods. Serial 2-fold dilutions of type 3 sera (●), type 5 sera (▲) and type 8 sera (■) and normal mouse sera (+) were tested for reactivity with heat-killed, formalin-fixed, type 8 bacteria using polyvinyl chloride (non-tissue culture treated) plates. Absorbance following ELISA analysis of antibody binding is presented on y-axis, while the reciprocal of the dilution of sera is presented on the x-axis.

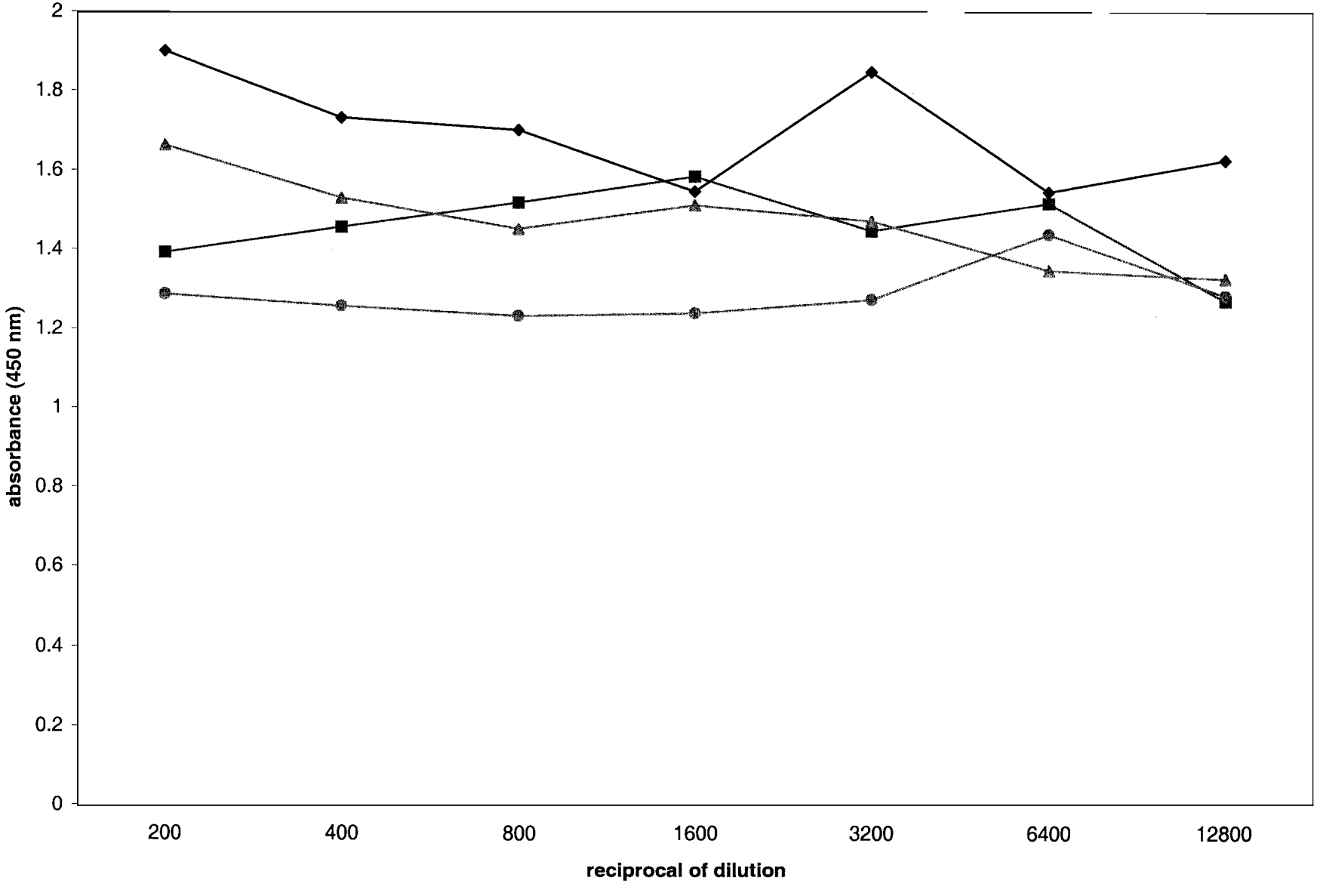

2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
absorbance (450 nm)
200
400
800
1600
3200
6400
12800
reciprocal of dilution

Figure 13. Binding of the Mouse Immunoglobulin to *Staphylococcus aureus* Type 8 Heat-Killed, Formalin-Fixed Bacterium; Assay Performed Using Flat-Bottomed Polystyrene Tissue Culture-Treated Culture Plates. Type 8 bacteria, that were used to coat the plates, were prepared as described in Methods. Serial 2-fold dilutions of type 3 sera (●), type 5 sera (A) and type 8 sera (■) and normal mouse sera (+) were tested for reactivity with heat-killed, formalin-fixed, type 8 bacteria, using tissue-culture treated plates. Absorbance following ELISA analysis of antibody binding is presented on y-axis, while the reciprocal of the dilution of sera is presented on the x-axis.

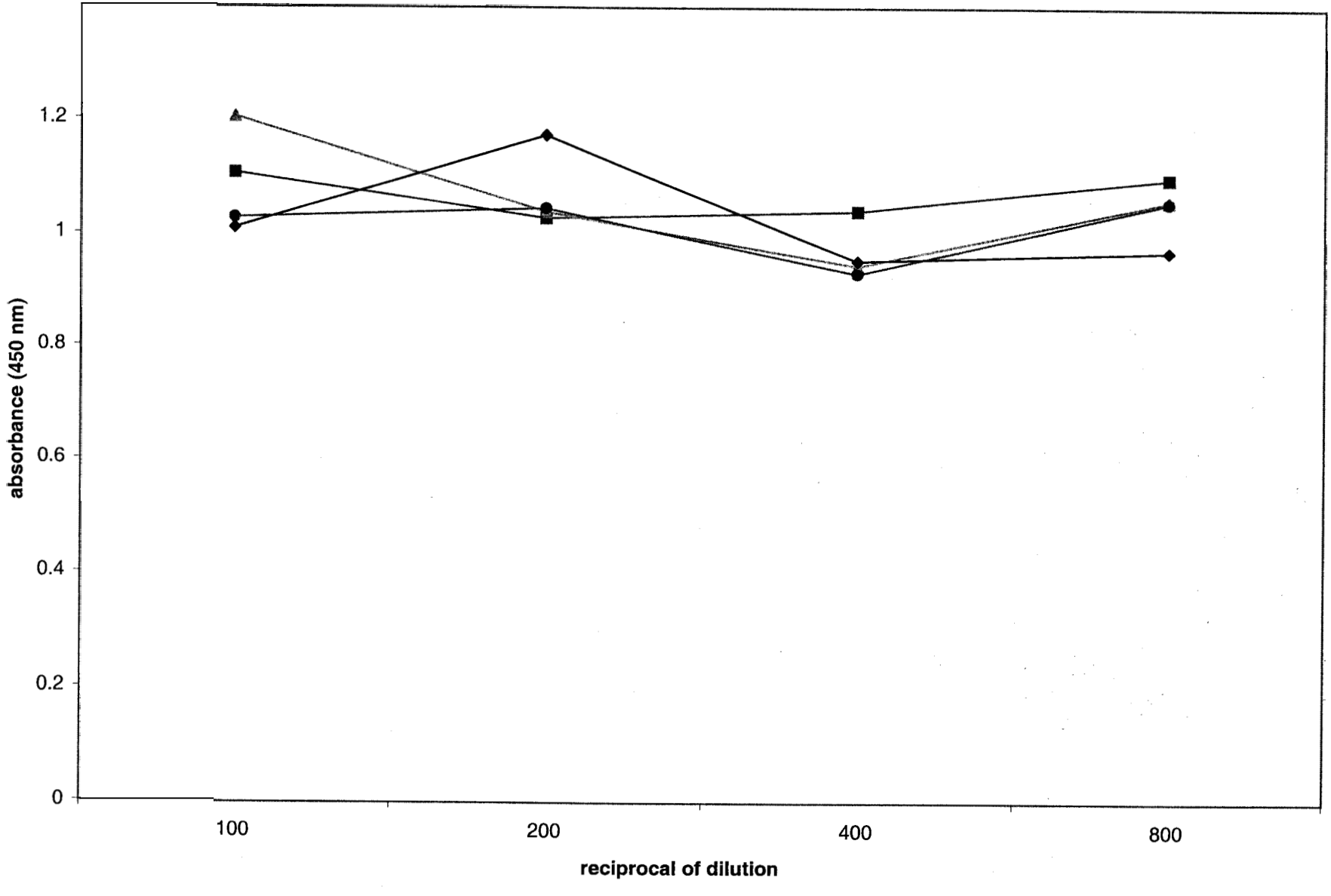
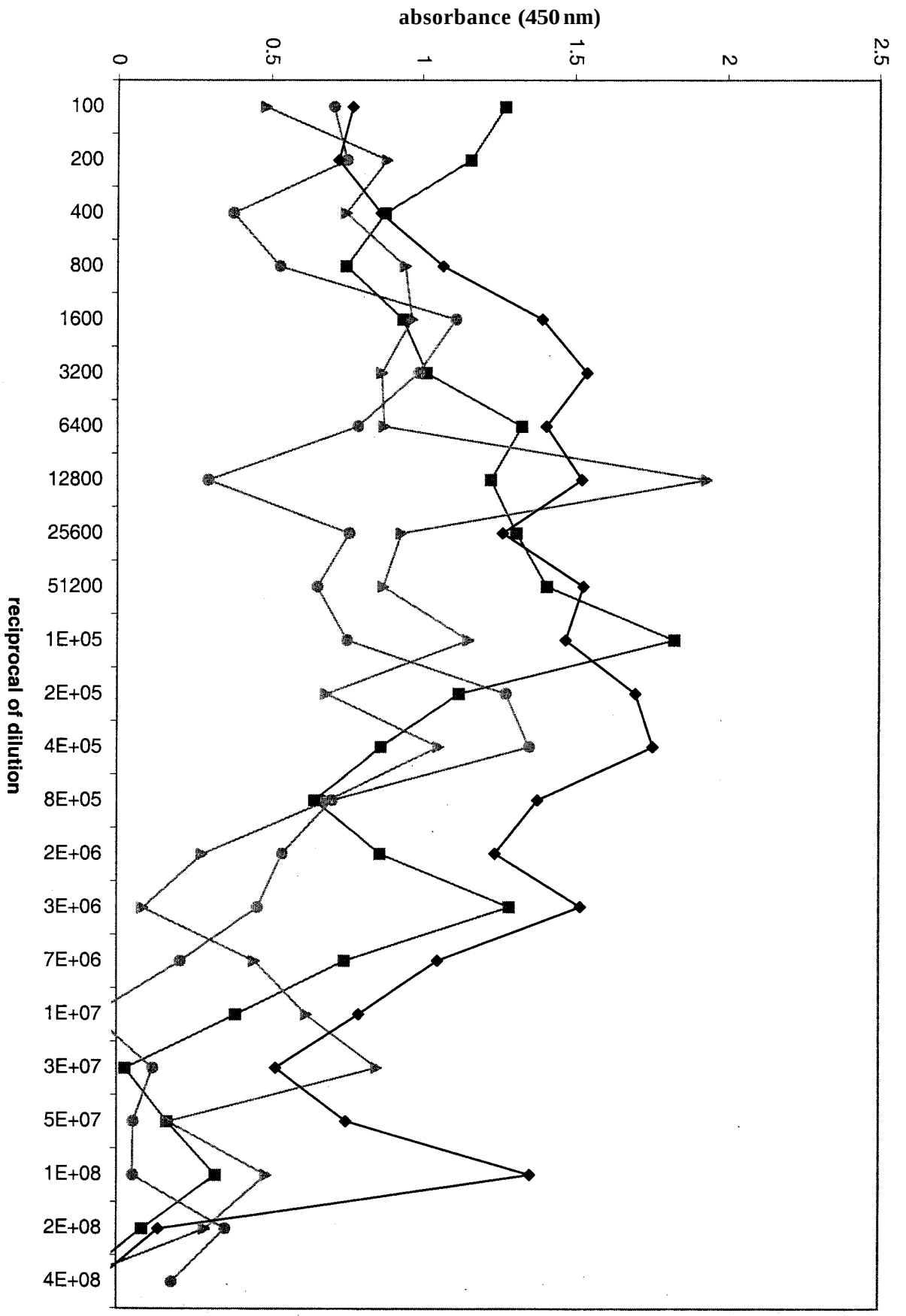

absorbance (450 nm)
1.2
1
0.8
0.6
0.4
0.2
0
100
200
400
800
reciprocal of dilution

Figure 14. Binding of Mouse Serum Antibodies to *Staphylococcus aureus* Type 8 Heat-Killed, Formalin-Fixed and Trypsin-Treated Bacterium. The bacteria were cultivated in 0.5 L Columbia Broth, supplemented with 2% NaCl. The pellet was treated overnight with 3% formalin at 4°C. After removal of formalin with PBS, the bacterial suspension (100 ml) was trypsinized with 1 mg/ml of trypsin, with a couple of drops of chloroform to help deproteination (Ueda et al, 1998). After overnight incubation at 37°C, and washing in PBS, the suspension was diluted to OD_{550}=1.0. The plates were incubated with 100 µl per well of this suspension, as described in Figure 12. Serial 2-fold dilutions of type 3 sera (●), type 5 sera **(A)** and type 8 sera (■) and 1 µg/ml of mouse immunoglobulin (+) were tested for reactivity with heat-killed, formalin-fixed and trypsin-treated type 8 bacteria. Absorbance following ELISA analysis of antibody binding is presented on y-axis, while the reciprocal of the dilution of sera is presented on the x-axis.

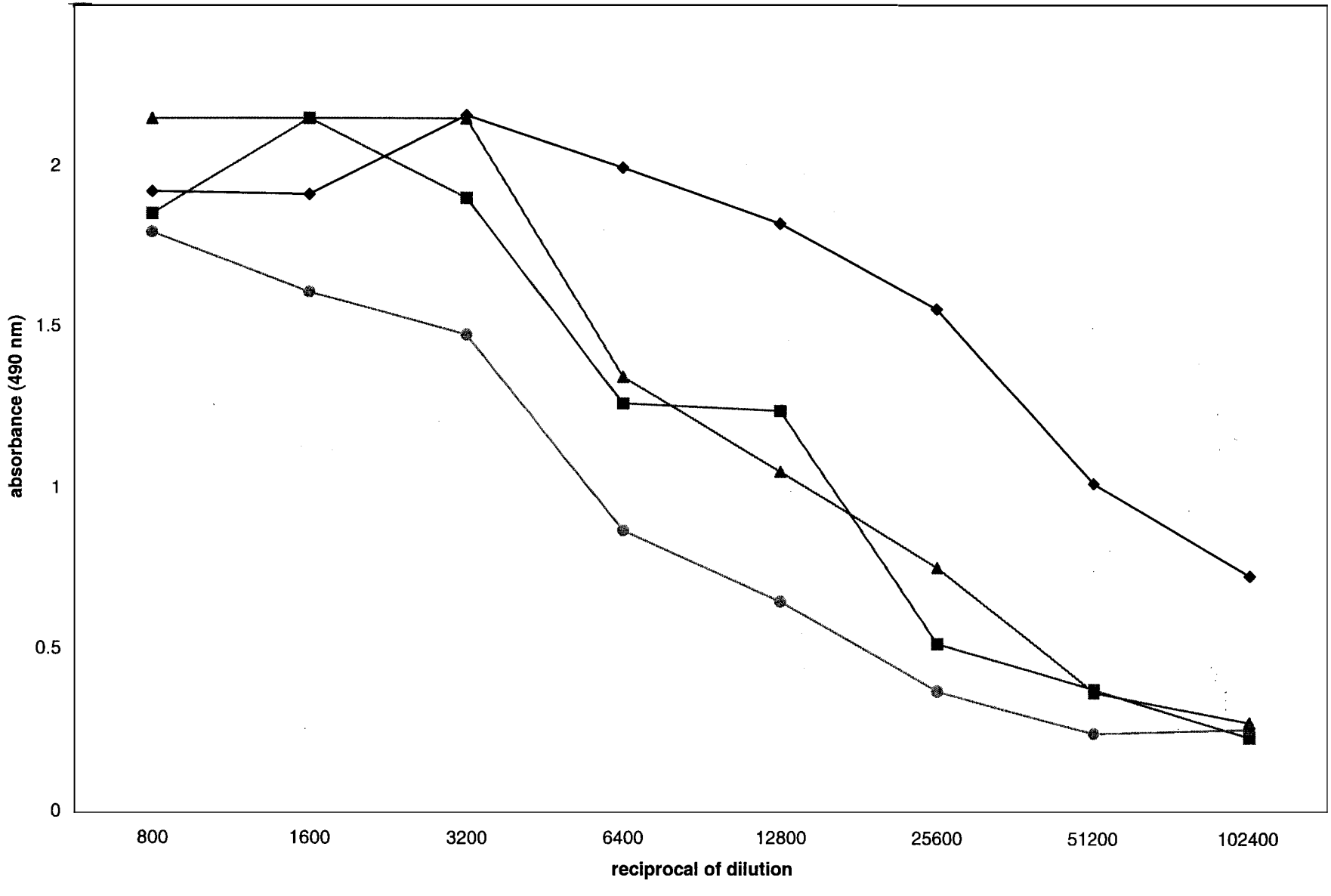

absorbance (450 nm)
0
0.5
1
1.5
2
2.5
reciprocal of dilution
100
200
400
800
1600
3200
6400
12800
25600
51200
1E+05
2E+05
4E+05
8E+05
2E+06
3E+06
7E+06
1E+07
3E+07
5E+07
1E+08
2E+08
4E+08

As whole cells were used in the assay, there was a possibility that the bacterial cells contained endogenous peroxidase activity that was affecting conversion of substrate to a colored form. Incubation of trypsin-treated cells with hydrogen peroxide (Jones and Gregory 1988), to inactivate any peroxidase present was therefore attempted (Fig. 15). After the incubation in the wells, and before the treatment with the blocking buffer, the cells were trypsinized and diluted in PBS. The wells were then treated with hydrogen peroxide prior to performance of ELISA. The ELISA results following trypsinization and hydrogen peroxide treatment were significantly improved. Specific antibody binding was deterrnined, as increasing dilution of serum resulted in decreased antibody binding. Type 8 serum, with a titer of 51200 and type 5 (titer = 25600) had much higher specific binding then type 3 (titer=12800). Unexpectedly, Mouse Ig also showed significant binding (titer more than 102400).

The same experiments were performed using type 5 heat-killed, formalin-treated whole cell bacteria to coat the 96-well polyvinyl chloride plate. The bacteria were treated with trypsin and hydrogen peroxide, as described previously, before being diluted in PBS to an OD_{550} of 1.0. ELISA results showed (Fig. 16) that the type 5 serum bound best to type 5 bacteria (titer

Figure 15. Binding of Mouse Serum Antibodies to Heat-Killed, Formalin-Fixed, Trypsinized and Hydrogen Peroxide-Treated Type 8 Bacteria. The bacteria were cultivated in 0.5L Columbia Broth, supplemented with 2% NaCl. The pellet was treated with 3% formalin overnight at 4°C. After removal of the formalin with PBS, the bacterial suspension (100 ml) was trypsinized with 1 mg/ml of trypsin, with couple of drops of chloroform, to aid in deproteination. After overnight incubation at 37°C, and washing in PBS, the suspension was treated with 3% hydrogen peroxide for 15 minutes (100 µl/well) in order to remove endogenous peroxidases; and diluted to OD_{550}=1.0. The plates were incubated with 100 µl per well of this suspension, as described in Figure 12. Serial 2-fold dilutions of type 3 sera (●), type 5 sera (▲) and type 8 sera (■) and 1 µg/ml of mouse immunoglobulin (+) were tested for reactivity with heat-killed, formalin-fixed, trypsin-treated and hydrogen peroxide treated type 8 bacteria. Absorbance following ELISA analysis of antibody binding is presented on y-axis, while the reciprocal of the dilution of sera is presented on the x-axis.

Figure 16. Binding of Mouse Serum Antibodies to Heat-Killed, Formalin-Fixed, Trypsinized and Hydrogen Peroxide-Treated Type 5 Bacteria. Type 5 bacteria were prepared as described in Figure 15. Serial 2-fold dilutions of type 3 sera (●), type 5 sera **(A)** and type 8 sera (■) and 1 μg/ml of mouse immunoglobulin (+) were tested for reactivity with heat-killed, formalin-fixed, trypsin-treated and hydrogen peroxide treated type 5 bacteria. Absorbance following ELISA analysis of antibody binding is presented on y-axis, while the reciprocal of the dilution of sera is presented on the x-axis.


absorbance (490 nm)
0
0.5
1
1.5
2
800
1600
3200
6400
12800
25600
51200
102400
reciprocal of dilution

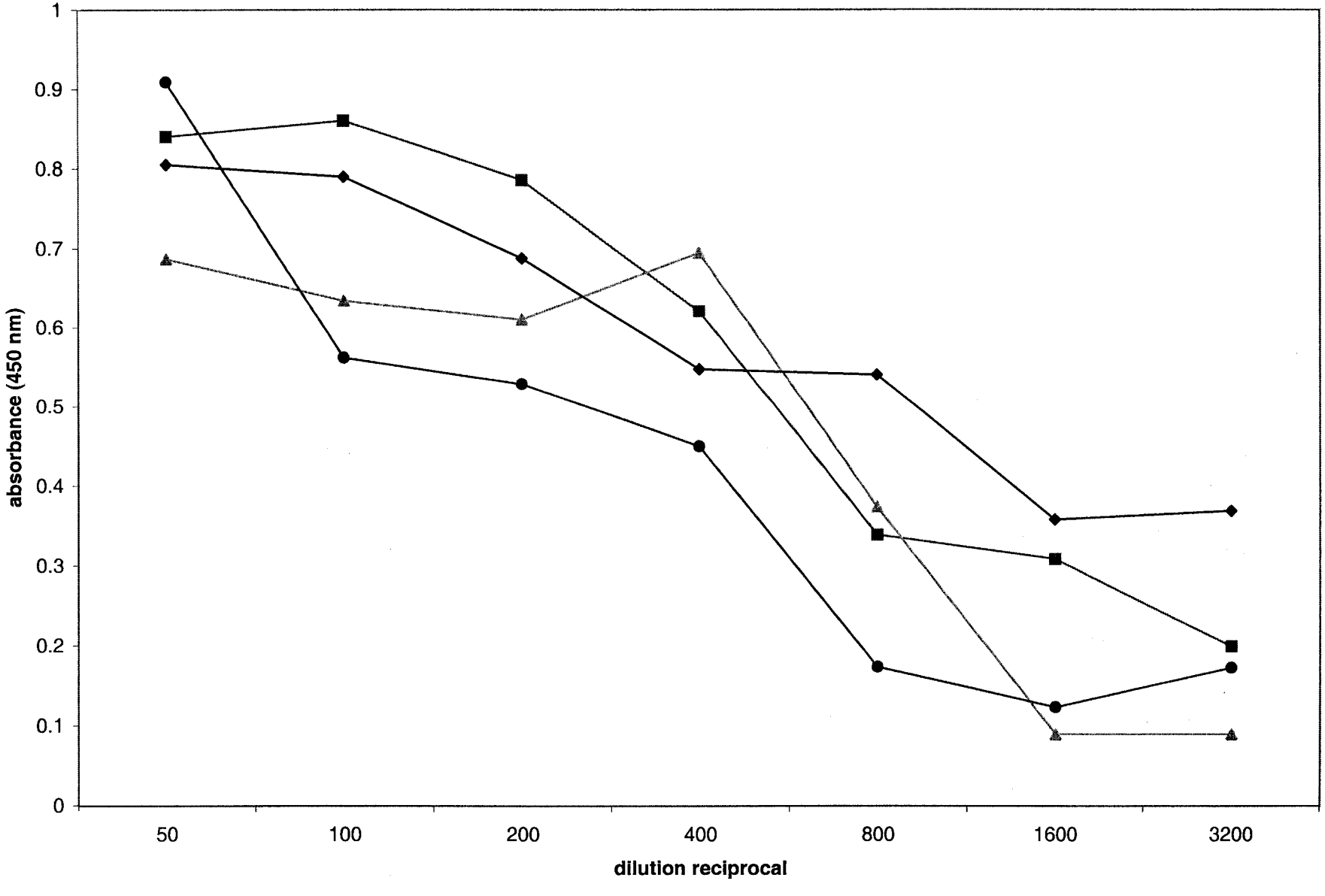
=38400), as expected. Type 8 binding (titer = 19200) was less then type 5, and type 3 serum bound the least (titer = 2400). Mouse Ig, again, had the highest binding of all three types of sera (titer more than 76800).

Binding of mouse serum antibodies to S. aureus serotype 8 purified capsular polysaccharide

As purified carbohydrate became available, an ELISA was developed using capsular polysaccharide, rather than whole bacteria. Sera from immunized mice were serially diluted across the 96-well plate that had been coated with 0.36 mg/well of type 8 purified polysaccharide (Fig. 17). Serum extracted from the mouse immunized with type 8 bacterium, showed the highest binding to the type 8 capsular polysaccharide (titer = 1600). Type 5 serum (titer = 800) did not seem to bind as well as type 8 serum to the type 8 CP, but much better than type 3 serum (titer = 400) which bound the least. Normal mouse sera (NMS), with a titer exceeding 3200, did not bind as well as type 8 serum up to the 1:400 dilution, but better than type 3 and type 5 at all dilutions.

The same sera (excluding NMS) were tested for binding to the 96-well plate coated with 0.11 mg/well of purified type 5 CP provided by Pam Massullo

Figure 17. Binding of Mouse Serum Antibodies to Purified Type 8 Capsular Polysaccharide. The plate was coated with purified capsular polysaccharide (0.37 mg/well). First well was added 1:50 dilution of sera from mice immunized with type 3 (●), type 5 **(A)** or type 8 (■) formalin killed whole cell bacterium, followed by the serial 3-fold dilutions across the plate. Pooled sera from normal mice were used as a negative control (♦). The protocol is described in Methods section. Absorbance following ELISA analysis of antibody binding is presented on y-axis, while the reciprocal of the dilution of sera is presented on the x-axis.


1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
absorbance (450 nm)
50
100
200
400
800
1600
3200
dilution reciprocal

(unpublished data) (Fig. 18). Maximum antibody binding for each serum was seen at all dilutions tested. Type 5 shows the highest binding at the 1:25600 dilution, at which point the ELISA microplate reader was unable to give a reading, because the assay was saturated. For the charting purposes, an absorbance value of 3.000 was assigned to this data point. Type 3 serum bound the least at all dilutions.

However, as serial dilutions did not show the expected decrease in binding, these results may have reflected nonspecific binding. the high carbohydrate concentration used may have caused the nonspecific binding. The same experiment was repeated using a smaller amount (1 μg/well) of the same type 5 CP (Fig. 19). In this experiment, Type 8 serum (with a titer of 400) showed better binding to type 5 CP (titer = 250) than the serum from mouse immunized with type 5 bacterium. Type 3 serum bound the lowest at the titer of 200, however it exceeded type 5 serum in the subsequent dilutions.

Binding of monoclonal antibodies to capsular polysaccharide

Antibody-prodycing hybridomas were produced from mice immunized with S. *aureus* Type 5 and Type 8 bacteria. However, only type 8 hybridomas showed growth. Monoclonal antibodies were selected for using limiting

Figure 18. Binding of Mouse Serum Antibodies to Purified Type 5 Capsular Polysaccharide (0.11 mg/well). Serial 2-fold dilutions of type 3 sera (●), type 5 sera **(A)** and type 8 sera (■) were tested for reactivity with purified type 5 capsular polysaccharide. Absorbance following ELISA analysis of antibody binding is presented on y-axis, while the reciprocal of the dilution of sera is presented on the x-axis.

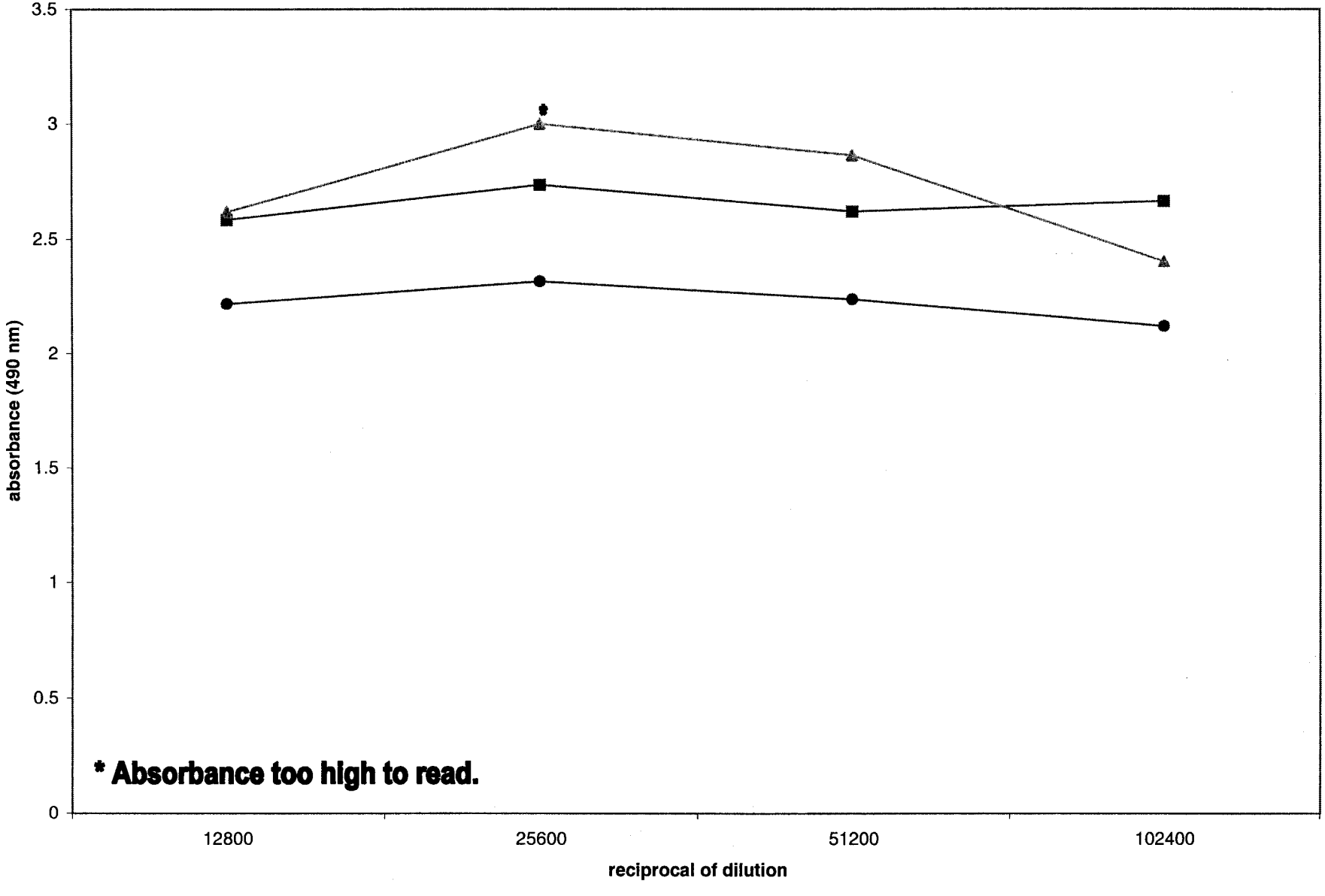
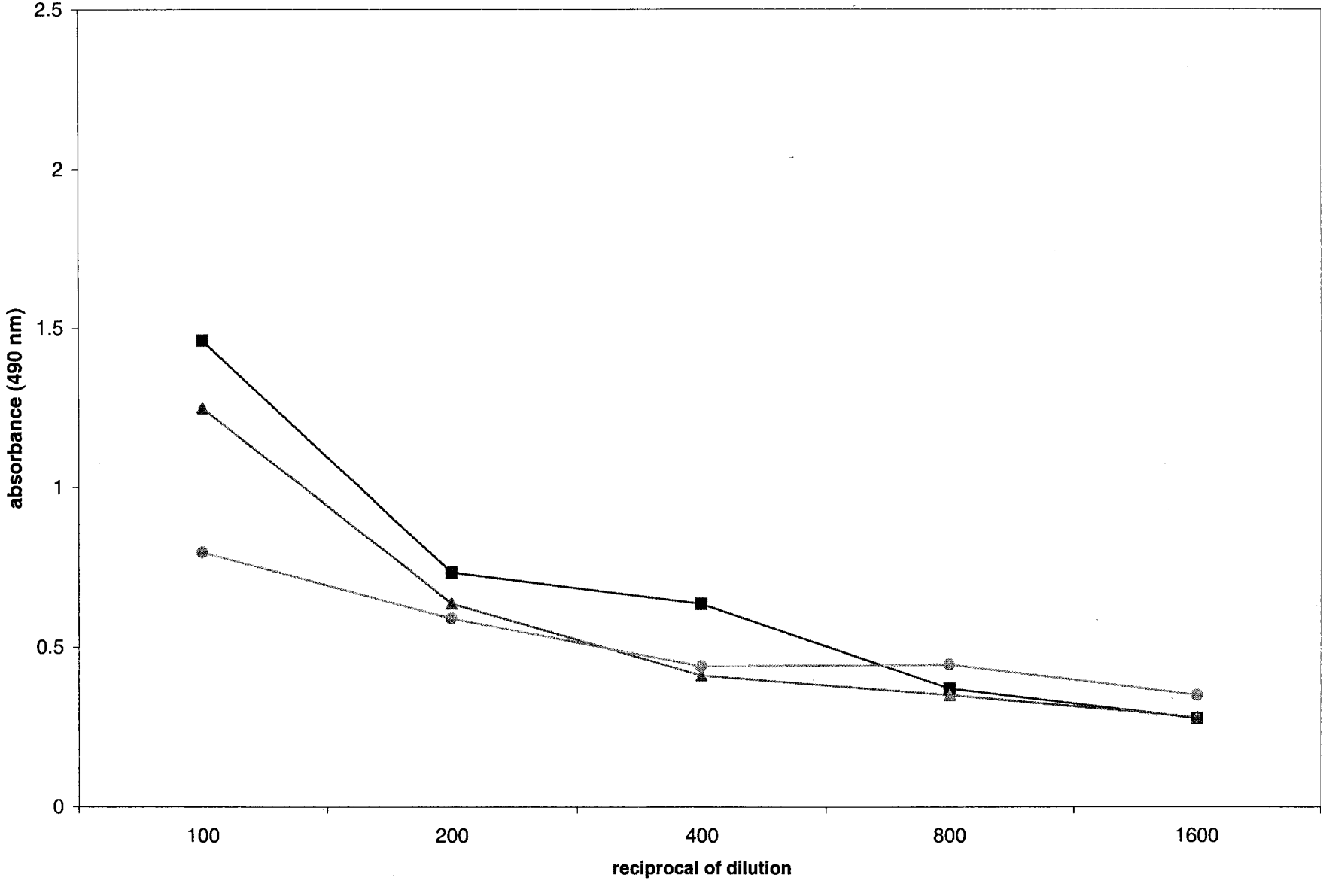

3.5
3
2.5
2
1.5
1
0.5
0
absorbance (490 nm)
12800
25600
51200
102400
reciprocal of dilution
* Absorbance too high to read.

Figure 19. Binding of Mouse Serum Antibodies to Purified Type 5 Capsular Polysaccharide (0.001 mg/well). Purified capsular polysaccharide (CP) was prepared as described in Methods. Concentration of CP that was used to coat the wells was 0.001 mg/well. Serial 2-fold dilutions of type 3 sera (●), type 5 sera (▲) and type 8 sera (■) were tested for reactivity with purified type 5 capsular polysaccharide. Absorbance of following ELISA analysis of antibody binding is presented on y-axis, while the reciprocal of the dilution of sera is presented on the x-axis.


2.5
2
1.5
1
0.5
0
absorbance (490 nm)
100
200
400
800
1600
reciprocal of dilution

dilution technique, and their binding to purified type 5 and type 8 capsular polysaccharide were tested by ELISA.

The results (shown in Table 1) indicate that none of the anti-type 8 monoclonal antibodies bound to type 8 CP. The exception was 1:4 dilution of T8.C2 antibody (value of 0.108), which was disregarder as a technical error because it was much higher then the 2-fold dilution of the same antibodies (value –0.055). Four of the anti-type 8 monoclonal antibody, however, bound to type 5 CP (values ranging from 0.037 to 0.127).

Table 1: Reactivity of Anti-Type 8 Specific S. *aureus* Monoclonal Antibodies With Purified Type 5 CP and Type 8 CP.

	Optical density value*	
Antibody	**Type 5 CP**	**Type 8 CP**
Tp.5 serum (1:10^3)	0.025	0.377
Tp.8 serum (1:10^3)	-0.118	0.352
Tp.3 serum (1:10^3)	0.05	0.443
T8.A6	0.033	-0.061
T8.B5	0.127	-0.046
T8.B5 (1:2)	0.037	-0.064
T8.B5 (1:4)	-0.032	-0.042
T8.B5 (1:8)	-0.067	-0.065
T8.B5 (1:16)	-0.059	-0.062
T8.B5 (1:32)	-0.07	-0.07
T8.B5 (1:64)	-0.046	-0.086
T8.B5 (1:128)	-0.069	-0.03
T8.B5 (1:256)	-0.007	0.029
T8.B4	0.117	-0.062
T8.D4.	0.095	-0.042
T8.C2	0.088	-0.068
T8.C2 (1:2)	0.054	-0.055
T8.C2 (1:4)	0.02	0.108
T8.C2 (1:8)	0.022	-0.052
T8.C2 (1:16)	-0.083	-0.053
T8.C2 (1:32)	-0.101	0.071
T8.C2 (1:64)	-0.135	-0.065
T8.C2 (1:128)	-0.115	-0.055

* Hybridoma culture supernatants (rows 4-20) and serum (rows 1-3) were added to the polyvinyl chloride plates coated with purified type 5 and type 8 capsular polysaccharide. The plates were incubated overnight at room temperature. Antibody binding was assessed with horseradish-peroxidase goat anti-mouse Ig in ELISA as described in the Methods.

V DISCUSSION

Purification and characterization of Type 8 capsular polysaccharide

In this study, Type 8 capsular polysaccharide (CP) from Staphylococcus aureus (S. aureus) bacteria was purified and characterized. This purification of Type 8 CP was initially done using the modified method of Fattom et al. (1990). Fattom and coworkers purified Type 5 and Type 8 CP by culturing the bacteria in 2% NaCl Columbia broth in a 100-liter fermentor at 37°C. Bacteria were harvested after 18-22 hours, as previous studies had determined that maximal capsule production occurred during this period (Karakawa and Kane, 1972). After the removal and washing of the cultivating broth, they killed the bacteria with phenol-ethanol solution, and collected the paste. This step required centrifugations at 25,000 x g, which was not possible for this study. When preliminary studies using this step were done using a slower speed, no detectable CP could be demonstrated in subsequent testing. This meant that too much of the CP was being released into the supernatant during the phenol-ethanol treatment, and it was not being collected with the slower centrifugation. The amount of bacteria used in this study was also drastically smaller than the previous study (9 liters vs. 100 liters), which also contributed to the smaller yield of CP. Alternatively,

a method developed by Fournier et al. (1984) was adopted. In this method, the bacteria were lysed by autoclaving at 121°C for 15 minutes. After centrifugation at 23,000 *x* g, the supernatant (containing the CP and other bacterial components) was collected. Lysis of the bacteria is a crucial step in the purification of CP, as Fournier and coworkers found that without cell lysis, CP couldn't be detected in the supernatant (Fournier et al., 1984). Only after the cells were lysed by autoclaving and/or when the capsule was penetrated with lysostaphin, were they able to detect the CP in the supernatant. Lysostaphin contains glycylglycine endopeptidase, which is used as a "probe of penetrability" of the *S. aureus* capsule, because of its ability to pass freely through the capsular layer (King et al., 1980). Once it crosses the capsule, it specifically lyses *S. aureus* cells by hydrolyzing bonds in the polyglycine bridges that form cross-links in the staphylococcal peptidoglycan (King et al., 1980).

At this point, CP and other bacterial components were in the supernatant. Fattom et al. adopted this technique, and continued further digestion with DNAse, RNAse and proteinase K to remove most of the nucleic acids and proteins, as also described in this study. This was followed by the CP purification using DEAE-Sephacel ion-exchange chromatography. DEAE (diethylaminoethyl) is a weak

anion exchanger used to separate the negatively charged carbohydrate (Karakawa and Kane, 1972) from proteins and neutral sugars (Robyt and White, 1987). This is accomplished using stepwise elution with increasing salt concentrations (0.05-0.5 M).

Once the CP was purified, it needed to be characterized. Fattom et al (1990) and Fournier et al. (1984 and 1987) detected CP in the eluate by a capillary precipitation test. This is a technique that uses rabbit anti-Type specific serum to precipitate the carbohydrate out of the solution. Capillary precipitation was used to detect the teichoic acid as well. Unfortunately, antibody was not available at the time of this study, to detect either CP or the teichoic acid, so alternative methods had to be searched for. Type 8 CP characterization, used in these studies was adopted from the studies done by Reynaud-Rondier et al. (1991) and Fieser and Williamson (1987) with modifications.

Reynaud-Rondier et al. (1991) monitored the presence of CP in their eluate using UV absorbance at 206 nm. They observed two peaks with this method, one peak contained protein and second peak contained primarily the carbohydrate. In our study, no prominent peaks were seen when the eluate

(from the DEAE-Sephacel column) was tested for the presence of CP, using absorbance at 206 nm. However, when the samples were analyzed using an overlaid sample spectra for wavelengths 180 thru 340 nm, it was clear that all of the individual fractions had components that were absorbing at 213 nm and 260 nm. The absorbance at 260 nm was disregarded because it detects the presence of nucleic acids (Fournier et al., 1984 and Gilbert et al., 1993). When all of the fractions were retested with a 213 nm absorbance, three peaks were seen in the fractions eluted with the buffer containing 0.15 M NaCl, and only one broad peak in the fractions eluted with the buffer containing 0.2 M NaCl.

The next challenge was to identify the peaks and separate the capsular polysaccharide from other components, such as teichoic acid and protein. Fournier et al. (1984) used gas chromatography and NMR analysis to test for the three compounds. Gilbert et al. (1993) used competition ELISA with rabbit anti-teichoic acid and anti-CP sera to detect CP and teichoic acid, and the absorbance at 260 nm to detect the protein. As antibodies to CP and teichoic acid were not available, Pam Massullo tested Red Tetrazolium sensitivity as a method for detecting reducing sugars, according to the method of Fieser and Williamson (1987). The Red Tetrazolium test worked

in the presence of the Tris acetate buffer used to elute the CP, and was the most sensitive CP detection method tested (Massullo, unpublished data). When the fractions from this study, collected from the DEAE-Sephacel column, were tested with Red Tetrazolium, both of the peaks contained reducing sugars and two additional peaks were indicated. The first peak contained two components (Fig. 10). The first part of the peak did not test positive for reducing sugars and was concluded to contain protein, as seen in the study by Reynaud-Rondier et al., mentioned earlier. However, the second part of the peak tested strong for reducing sugar and was labeled peak # 2.

Once the protein peak was excluded, reducing sugar-containing peaks needed to be tested further for the presence of teichoic acid. Peaks that tested negative for teichoic acid (and positive for reducing sugars) would be considered as the CP peaks. As gas chromatography or NMR analysis were not readily available, Pam Massullo tested a phosphate test, developed by Chen et al. (1956), that would detect the presence of phosphate contained in the teichoic acid. During the testing, peaks 1 and 2 were combined by mistake (fractions 10-25) from the buffer containing 0.15 M NaCl. When the fractions from each individual peak were tested, only peaks 1 and 2

(combined); and peak # 3 tested free of phosphate (Fig. 10). This CP was then used to test for binding to the mouse CP-specific sera, using enzyme linked immunosorbent assay.

Detection of mouse serum antibody to inactivated S. aureus (Type 3, 5 and 8) whole-cell bacteria.

In an attempt to produce a more specific antibody test for the detection of CP in the mixture, mice were immunized with heat-killed, formalin-treated Type 5, Type 8 and Type 3 bacteria. Type 3 serum was used as a negative control, because Type 3 S. *aureus* is indicated in the literature as being serologically distinct from Type 5 and Type 8 S. *aureus*. Thus, it was assumed that Type 3 CP, whose structure has not yet been chemically characterized (Lee and Lee, 1999), has different antibody binding sites from Types 5 and 8, and would make a good negative control. Mouse serum antibodies were tested for their binding to Type 5 and Type 8 whole bacteria to develop a method for identifying the antibodies against the CP without using as yet unpurified CP to coat the plates. Mouse sera, specific for Type 5 and Type 8 bacteria, successfully bound to the whole bacteria. Type 3 sera reacted weakly with both Type 5 or Type 8 bacteria. This shows that the epitopes of Type 5 and Type 8 strains are different from strains producing type 3 CP, and that Type 3 antisera was a good negative control. However,

cross-reactivity between the Type 5 and Type 8 sera was seen throughout the study.

The fact that the two capsules are listed as distinct serotypes suggests that, at least with rabbit antiserum, they are immunologically distinct strains. However, controversial reports exist on the cross-reactivity between Types 5 and 8 in the literature. For example, Nelles et al. found that Type 5 and Type 8 mouse monoclonal antibodies showed no cross-reactivity in ELISA, when tested for their binding with variety of gram-positive and gram-negative bacteria (1985). On the other hand, Karakawa et al. (1972) using immunoelectrophoresis showed that there is a slight cross-reactivity in surface antigens between different S. aureus strains. The reason for the crossreactivity might be the fact that Type 5 and Type 8 share certain proteins on the surface of the cells. If these proteins are present in the carbohydrate preparations, they may bind to antibodies produced against either strain of bacteria. However, our studies suggest that protein contamination was not a problem, as Type 3 antisera did not bind to Type 5 or Type 8 CP. If proteins were present, Type 3 antisera should have bound.

It has also been shown that other organisms, such as *Neisseria gonorrhoeae* (Hutchinson, 1970) or *Escherichia coli* (Ward, 1970), lose some of their surface antigens during cultivation. S. *aureus*, as well, may be susceptible to the loss of antigens due to different culture conditions. Further investigation is needed to see if this is the reason that some research groups get cross-reactivity between the strains, and others do not.

Pooled sera from the unimmunized mice (NMS), as well as the pooled antibodies from unimmunized mice (mouse Ig) showed the presence of antibodies against both Type 5 and 8 bacteria and CP. The results were not all that surprising, taking into the consideration the ecology of S. *aureus.* Since this bacterium frequently colonizes both animal and human populations, it is likely that some of the sera (used to make the pooled NMS, or used to purify the immunoglobulin from) had come from the animals that have had a staph infection. Our results suggest that both of the suspensions (NMS and mouse Ig) already contained antibodies against Type 5 and Type 8 bacteria, which were detected in ELISAs.

The antibodies tested in this study were polyclonal (made by more then one B cell clone) and, thus were able to bind to many different epitopes

(antibody binding sites) on the CP. Type specific, rabbit antiserum, containing polyclonal antibodies, has been successfully used before to detect the presence of Type 5, Type 8 and other Types of S. aureus, using imrnunoelectrophoresis (Arbeit et al., 1984). Furthermore, polyclonal antiserum has been used in ElLISAs designed to quantitate the amount of anti-Type 5 CP antibodies from mice immunized with the live Type 5 S. aureus (Thakker et al., 1998). Some of the problems of cross reactivity may be related tot he use of polyclonal, rather than monoclonal antibodies.

Detection of mouse sera antibody to inactivated S .aureus (Type 3, 5 and 8) capsular polysaccharide.

ELISA results also showed that at very high concentrations of Type 8 capsular polysaccharide, there are a large number of antibodies specific for Type 8 CP, as expected. Serum from the mouse immunized with Type 5 bacterium also proved to contain antibodies that reacted with Type 8 CP. It is possible that some of the whole bacteria, used to immunize the mice with, could have had a damaged capsule (exposing the teichoic acid), which would stimulate the production of antibodies against the teichoic acid. Furthermore, CP preparations may have been contaminated with teichoic acid, which would react with the antibodies from the serum. Since both Type 5 and Type 8 bacteria contain teichoic acid, then their antisera would have

the same antibodies, which would show up in ELISA. (Similar results were obtained with ELISA plates coated with Type 5 CP.) Similarly, previous studies attributed any cross reactivity between Type 5 and Type 8 sera in agglutination assays, to the presence of anti-teichoic acid antibodies (Sutra et al., 1990 and Sompolinsky et al., 1985). It is important to add that Type 3 bacteria also has teichoic acid in their wall, but showed no cross reactivity with either Type 5 or Type 8 CP. This led us to believe that teichoic acid contamination is just a part of the reason for the cross-reactivity between the Type 5 and Type 8 CP and that there may actually be cross-reactive epitopes when testing mouse sera. However, the ELISA methods developed here demonstrate that antibodies to other seroTypes (Type 3) were not able to bind to Type 5 or Type 8 CP. The ELISAs developed could be useful as a rapid method of detection of antibodies to CP in future studies to develop monoclonal antibodies to CP.

In summary, ELISAs using whole bacteria showed better binding of the specific sera than of those ELISAs using purified CP. The reason for this may be due to contaminants being detected in the CP. The same contaminants may not be seen as much in whole bacteria assays, because the capsule is still intact and covering up other antigens.

Boutonnier et al. (1989) have investigated the specificity of the ELISA detection of S. *aureus* CP in blood cultures, using monoclonal antibodies. They saw no cross reactivity between the Type 5 and Type 8 anti-CP monoclonal antibodies. Therefore, the most likely way to eliminate any cross binding between the Type *5* and Type 8 antisera, would be to produce monoclonal antibodies, that would bind to an epitope specific for each type.

Production of monoclonal antibodies specific for Type 8 bacteria.

Since the sera from immunized mice failed to clearly distinguish Type 5 and Type 8 capsular polysaccharide in our studies, production of type-specific monoclonal antibodies was initiated in an attempt to eliminate the cross-reactivity between the sera.

Attempts to isolate Type specific monoclonal antibodies against different seroTypes of S. *aureus* have been made previously (Nelles et al., 1985). In the studies by Nelles and coworkers, anti-Type 5 and anti-Type 8 monoclonal antibodies were made by fusing the spleen cells of mice immunized with whole cell heat-killed, formalin-fixed bacteria (Type *5* and Type 8), with a mouse myeloma fusion partner, X63-Ag8.653. After selecting for antibody-producing-hybrids with HAT, individual hybrids

(producing monoclonal antibodies) were separated by limiting dilution. To see if the monoclonal antibodies produced were directed against *S.* aureus, an agglutination assay was performed, using suspensions of heat-killed, trypsinized organism. Only those hybridomas that agglutinated the homologous strain (used as an immunizing agent) were analyzed further. In their studies, ELISA using whole cell, formalin-fixed bacteria showed that monoclonal antibodies produced from mice immunized with S. aureus Type 5, bound only to the Type *5* bacteria, as expected. Hybridomas made with spleen cells from the mouse immunized with Type 8, also only bound to Type 8 bacteria. However, when the ELISA was performed using capsular polysaccharide instead of whole cell bacteria, different results were seen. In these assays monoclonal antibodies specific for Type 8 *S.* aureus reacted strongly with Type 8 CP. However, they were unable to show that anti-Type *5* monoclonal antibody bound to the purified Type *5* CP, using the same method.

In our studies, the production of monoclonal antibodies to both Type 5 and Type 8 was attempted. Mice were immunized with formalinized bacteria, and their sera was tested using purified CP from Type 5 and Type 8 *S.* aureus. Fusions of the spleen cells from the mouse immunized with Type *5*

bacteria were unsuccessful (no clones were established). Out of 288 wells containing spleenocytes from the Type 8-immunized mice, four showed growth. All four were subcloned using limiting dilution method, and then tested for binding to S. aureus Type 5 and Type 8 CP. Anti-Type 8 hybridomas, produced in this study, did not prodece antibodies that bound to the Type 8 CP. However, four anti-Type 5 monoclonal antibodies bound to the Type 8 CP.

The two clones tested had only been subcloned once and may still represent more than one B cell clone. However, the results suggest that crossreactivity between the two capsular Types may still be seen with monoclonal antibodies. While this would make it more difficult to identify the capsular Type being produced by S. aureus, it would be an advantage therapeutically. One antibody that reacts with two of the predominant disease causing strains of S. *aureus* would simplify treatment strategies. Further work needs to be done to develop and test more clones producing antibodies to S. aureus capsular polysaccharide.

Summary

In this study Type 8 *S. aureus* capsular polysaccharide was successfully purified and new methods were developed for its characterization. It was shown that CP presence can be monitored using absorbance at 213 nm, followed by detection of carbohydrate with a Red Tetrazolium test. Microdetermination of phosphorus allowed teichoic acid contamination to be detected, thus indicating peaks containing pure CP.

Sera from the mice immunized with Type 5 and Type 8 whole bacteria, successfully bound to the Type 5 and Type 8 CP (and the trypsin and hydrogen peroxide treated bacteria) respectively. However, the antibodies in the serum were still polyclonal, and cross-reactivity between antibody binding to Type 5 and Type 8 CP was seen. Following hybridoma production, Type 8 antibodies failed to bind to the Type 8 CP, but three of the Type 5 hybridomas bound the Type 8 CP.

Future studies will involve developing more monoclonal antibodies and testing for those that bind specifically to a particular Type. These

monoclonal antibodies can then be used to develop a rapid and highly sensitive test for the detection of infections caused by *Staphylococci* in patients. Moreover, the monoclonal antibodies could be used for passive immunizations against staph infections in immuno-compromised patients. However, in humans, mouse monoclonal antibodies are seen as foreign molecules and stimulate the immune system. THis would result in ineffective treatment of the disease, as the patients immune response would eliminate the therapeutic antibodies. The availability of hybridomas would allow for extraction of the antibody genes and the manipulation of the genes to make them more humanized. Furthermore, antibody-gene manipulation, such as inducing random mutations in gene sequences would allow for the selection of an extremely tight-binding antibody, as well as allowing for linking the gene that codes for the antibody with a gene that codes for an antibacterial protein. This Type of antibody would have antibacterial properties and potentially be effective in the treatment of the infections caused by *S. aureus*.

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