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ANALYSIS OF IMMUNODOMINANT EPITOPES OF *NEISSERIA GONORRHOEAE* LIPOPOLYSACCHARIDE USING A COMPETITIVE INHIBITION - ENZYME LINKED IMMUNOSORBENT ASSAY

By

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Thesis

Submitted to the Faculty of Graduate Studies

in partial fulfillment of requirements for

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Department of Microbiology and Immunology

Faculty of Medicine

University of Ottawa

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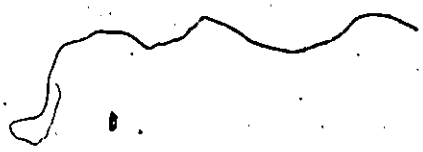

To Fred, June and Courtney

; love

like water

tumbles downhill

between generations.

A Mass for the Dead.

William Gibson

Table of Contents

List of Abbreviations

BSA	bovine serum albumin
CI-ELISA	Competitive Inhibition - Enzyme Linked Immunosorbent Assay
DNA	Deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
KDO	3-deoxy-D-manno-octulosonic acid
LPS	Lipopolysaccharide
MLPS	Lipopolysaccharide activated with methyl para-hydroxybenzimidate H Cl
MW	molecular weight
O-side chain	oligosaccharide side chain
OMP	outer membrane protein
PBS	phosphate buffered saline
PBS8	phosphate buffered with 8% NaCl
RIA	Radioimmunoassay
R type	rough type (lipopolysaccharide)
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
Seph-Ab	Sepharose Protein A coated with antibody
S type	smooth type (lipopolysaccharide)

Objectives

The first objective of this research was to assess the usefulness of the recent (1976) but increasingly used technique of enzyme linked immunosorbent assay (ELISA) as a tool for analyzing the immunodominant epitopes of Neisseria gonorrhoeae lipopolysaccharide. This would be achieved by comparing the ELISA test system to the classical immunoprecipitin test.

A second objective was to determine if the same epitopes evoked a similar antibody response in two different species of animals. This would be tested by comparing the antiserum obtained from capons with the monoclonal antibodies derived from mice.

Introduction

Gonorrhea is a major pandemic venereal disease, with an estimated 200 million cases worldwide in 1974 (1). It is the second most common infectious disease in the United States, first among notifiable diseases (2) and a major cause of sterility and infertility (2).

Jessamine et al. (3) note that the reported number of cases of gonorrhea in Canada for 1981 was 56,000, which is approximately 30% of the total number of cases. They further note that the total number of cases of sexually transmitted diseases was in excess of 1 million. The economic costs of uncomplicated sexually transmitted diseases in the late 1970's is reported to exceed 200 million dollars annually. All factors being equal this extrapolates to a cost of 11.2 million dollars for treatment of reported uncomplicated gonococcal infections annually in Canada. This does not take into account indirect costs of complicated infections which include pelvic inflamatory disease for which hospital separation rates tripled between 1961 and 1977, and ectopic pregnancy which resulted in 22,860 deaths between 1971 and 1978 (3). Also included in indirect costs are resultant infertility, time lost from work and human suffering.

Immunity to gonorrhea is transient or limited as evidenced by repeated infections, chronic conditions and carrier states. There is great antigenic variation among gonococcal strains and sharing of antigens with other Neisseria species (4). Consequently, there is an interest in the cell surface antigens of Neisseria gonorrhoeae, particularly on the role of such antigens in the pathogenesis of, and immunity to, this organism.

There are three major groups of cell surface antigens on the outer surface of N. gonorrhoeae which might evoke an immune response. These are: the pili, outer membrane proteins (OMP) and lipopolysaccharides (LPS). While they obviously act in unison with one another, in the host-organism interplay they are most easily studied as separate distinct groups.

The LPS molecule was chosen as the antigen for this study for several reasons:

1. Unlike the other antigens (pili, OMP) it is common to all N. gonorrhoeae studies to date (5,6).

2. It is well defined chemically and is easily obtained in large quantities.

3. The antibody - antigen interaction has been studied previously (7), providing a sound basis for further study.

In this thesis it is shown that the competitive inhibition enzyme linked immunosorbent assay (CI-ELISA) is a rapid and reliable test for the detection of immunodominant epitopes of the N. gonorrhoeae lipopolysaccharide molecule.

The work of Perry et al (7) demonstrating that 4-O-(β-D galactopyranosyl)-D-glucopyranose, commonly known as lactose, is a major immunodeterminant of gonococcal LPS, is confirmed using the CI-ELISA technique. In addition it is demonstrated that the monoclonal antibodies derived from mice and polyclonal capon antiserum recognize the same portion of the LPS molecule.

Characterization of N. gonorrhoeae Cell Surface Antigens

PILI

In 1963 Kellogg (8) developed a typing scheme for N. gonorrhoeae based on the size, texture and transparency of the colonies grown on a transparent medium. The major colony types were T1, T2, T3, T4. T1 colonies are darkgold, convex, translucent, amorphous and viscid. T2 colonies are darkgold, convex, translucent, amorphous, friable, with crenated edges. T3 colonies are light brown, low convex, translucent, granular and viscid. T4 colonies are colorless, low convex, transparent, amorphous and viscid. Subsequently he showed (4), using human volunteers, that only the T1 and T2 types were virulent in humans. Later Swanson (9) and Jephcott et al. (10) both demonstrated that these two colony types had pili while T3 and T4 did not. It has also been shown that the colony types revert from one to another with a frequency dependent on the strain and composition of the typing media (11).

The pili are filamentous protein fibers extending from the surface of the cell. Salit et al. (12) demonstrated heterogeneity of pili in 10 of the 14 opacity variants which were studied. These opacity variants are an extension of the original typing scheme and are dependent on outer membrane protein composition; they will be discussed in the section

devoted to outer membrane protein. The opaque variants exhibited a total of 7 distinct pili while the transparent variants had a total of 10 different pili. The pili were characterized by molecular weight, amino acid composition and immunologically. Most strains had 2 pili types present at one time.

Buchanan et al. (13) determined that pili are composed of a single protein subunit, 19,000 to 25,000 in molecular weight, varying with the strain tested. Pili have a density of 1.29 to 1.33 gm/cm^3 and are composed of 55% hydrophobic and 45% hydrophilic amino acids with approximately 2% carbohydrate by weight (13). The N-terminal sequence, when compared to other Gram negative bacteria, is unusual. The first 24 residues are all hydrophobic amino acids with the exception of threonine and glutamic acid in positions 2 and 5 respectively, from the N-terminal end. The N-terminal amino acid is N-methyl phenylalanine. The sequence of amino acids is identical to that of pili isolated from Neisseria meningitidis and Moraxella nonliquificans. Since all these microorganisms inhabit mucosal surfaces it would seem that this sequence may play a role in the interaction with the host. This sequence is also likely to be the cause of the 10% shared antigenicity between pili types noted by Buchanan et al. (13).

Punsalang and Sawyer (14) noted that T1 cells were significantly more resistant to phagocytosis by

polymorphonuclear leucocytes than were T4 cells. Depiliation reduced this resistance to phagocytosis. Ofek et al. (15) demonstrated that anti-T1 whole cell antiserum increased phagocytosis of T1 but not T4 cell types. Jones et al. (16) noted that non- piliated cells were phagocytized in the absence of antibody in heat inactivated normal rabbit serum. They also showed that opsonic antibody is absorbed from serum equally well by pili or whole organisms, but not by either outer membrane proteins or lipopolysaccharide.

Attachment to epithelial cells is inhibited by pili but not by LPS (17, 18). Swanson (19) demonstrated that a 3 to 8 times advantage is conferred to piliated over non-piliated strains in binding to cell membranes, depending on the origin of the membrane. However, he also noted that pili are not essential for binding to occur.

Buchanan (20) has shown that piliated cells compete more efficiently for iron, than do non-piliated cells. Several researchers (21, 22) have shown that iron is necessary for, or increases the virulence of bacteria.

Koransky (23) has shown that piliated gonococci agglutinate human erythrocytes, regardless of ABO blood group, but fail to agglutinate red blood cells of six other species.

Pili are a virulence factor of gonococci as evidenced by the piliated cells resistance to phagocytosis and increased attachment to mammalian cells. Although gonococcal pili have a common determinant the latter is shared with other organisms, limiting therefore its effectiveness in a diagnostic test.

Outer Membrane Proteins

Johnston (24, 25) developed a serotyping system based on the outer membrane protein (OMP) complex extracted from gonococci with lithium chloride. The majority of the gonococcal strains may be classified into sixteen serotypes. The serotype is stable on subculture and is consistent between contacts having gonorrhea. The outer membrane complex is composed of a major protein which accounts for up to 60% of the total protein in the complex, one or two co-variant proteins and LPS. More recently, Buchanan (26) has shown that 99% of the isolates tested belong to one of nine main serotypes, based on the major protein (protein I) in the outer membrane complex.

The OMPs are responsible for the changes in opacity noted in the various colony types (27). The major protein is found in all opacity variants. The major protein (protein I) is 36,000 MW and is found in all variants of strain P9. It is suggested that it is a trans-membrane protein with a similar function to that of porin in Escherichia coli, i.e. it may act as a hydrophobic diffusion pore (28).

Lamden et al. (29) identified the opacity related proteins of strain P9 and designated them II*, IId*, IIa*, IIb*, and IIe*. They have respective molecular weights of 29,000, 28,850, 28,500, 28,000 and 27,500. All the variant strains have a greater resistance to the bactericidal action of normal serum than does the transparent strain which has only protein I. Protein IIa* was responsible for the greatest

increase in serum resistance. Protein II* gave increased resistance to low molecular weight antimicrobial agents. Increased adhesion to human buccal cells was demonstrated by each opacity variant; piliation caused the greatest increase in adhesion. The transparent strain demonstrated the greatest number of cells adhering to erythrocytes, with pili further increasing the number of adhering cells. Protein IIa* gave increased association to leukocytes, IIb* decreased association, while pili had no effect. This demonstrated that adhesion to different cell types is mediated by various receptor sites.

Hildebrandt and Buchanan (30) have shown that 75% of strains isolated from disseminated gonococcal infections had a particular protein I, while only 30% of strains causing localized infection had the same protein I. The strains from disseminated infection had an increased serum resistance. The DNA of resistant stains could transform serum sensitive stains into serum resistant strains. Serum resistance in this case was mediated by a 36,000 MW surface protein. Lamden et al. (29) attributed serum resistance to a 28,500 MW protein. Therefore, different proteins may confer similar attributes to a cell and all the surface proteins may interact in defining certain properties.

LIPOPOLYSACCHARIDE

The LPS molecule is generally composed of three distinct portions: The lipid A, core polysaccharide and an oligopolysaccharide side chain. The hydrophobic lipid A portion is linked to a core polysaccharide through a 3-deoxy-D-manno-octulosonic acid (KDO) trisaccharide. The core portion is composed of these three KDO molecules linked to approximately 5 to 7 saccharides and is usually common to a species (31). This molecule is called rough LPS (R-LPS). A further O-side chain of repeating polysaccharide units may be attached to the R-LPS to form smooth LPS (S-LPS). The O-side chain is responsible for the serotype specificity of enteric bacteria (30). This structure is similar in most Gram negative organisms: O-side chain--core$(KDO)_3$--lipid A.

The 'smooth' and 'rough' designations come from early work with salmonellae. The naturally occuring isolates when cultured in vitro had a glossy, smooth appearance, which on repeated subculturing would become dry and wrinkled. The R colony types were found to be missing the O-side chain which was present in the S colony type.

The lipid A inserts into the membrane of the cell. It is this portion of the molecule which is responsible for the endotoxic and anti-complementary properties of the LPS. The lipid A portion is usually non-immunogenic, however, Galanos et al. (32) have produced rabbit antibody to purified lipid A by using as antigen purified lipid A absorbed to a Salmonella rough mutant. This antiserum is highly crossreactive with many

Gram negative species. The antigenicity of the polysaccharide core and O-side chain is increased by the adjuvant effect of the lipid A.

N. gonorrhoeae and N. meningitidis contain only the R type LPS; no smooth type LPS has been consistently demonstrated for either species (5, 32). The core portion of all gonococci studied to date has a similar composition and structure as determined by nuclear mass resonance spectra (31).

The core composition of many Neisseria species has been determined (31) (Table 1). It is interesting to note that the pathogenic species all have a similar composition while non-pathogenic species have an unrelated structure. Most non-pathogenic species are missing either D-galactose or N-acetyl-D-glucosamine or both from their core structure, whereas, the pathogenic species have both.

Tsai and Frasch (34) have reported on the SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) profile of meningococcal LPS. A double band is evident at the running front of the gel when stained with silver stain. It was determined that both bands are always present but their ratios may be altered by culture conditions. The difference in the bands is the inclusion of an extra galactose residue in the slower moving band. Similar banding patterns have been noticed in SDS-PAGE patterns of gonococcal LPS in the laboratory of Perry (unreported results). This is evidence of a microheterogeneity within the gonococcal LPS.

LPS may have several different immunological effects on the host (35):

1. Induces DNA synthesis in B cells leading to a proliferation of B cells.
2. Stimulation of immunoglobin secretion in spleen cell cultures.
3. Production of anti-DNA and other auto-antibodies in mice.
4. Supression of an immune reponse to a second antigen injected a few days after the injection of LPS.

LPS induces mainly an IgM response in rabbits. Only after repeated injections can IgG be detected, but the latter has a low titer and is less specific than the IgM (35). Ruttkowski et al. (37) and Karch et al. (38) report that LPS alone induces an IgM response but if administered in liposomes or embedded in membrane vesicles it evokes both IgM and IgG responses.

Normal serum is bactericidal for most strains of gonococci. The isolated LPS of resistant strains can absorb out the bactericidal activity of the serum so that sensitive strains are no longer killed in the presence of the absorbed serum. LPS isolated from serum sensitive strains will not absorb out this anti-bacterial activity.

Guymon (39) has shown that normally serum sensitive strains can be converted to serum resistant when transformed with the DNA of an originally resistant strain. Further characterization of the change in LPS has not been done.

Wallace et al. (40) and Malysheff et al. (41) have shown that either hen or capon antiserum directed to the gonococcal LPS molecule agglutinates approximately 99% of the gonococcal strains tested, with minimal cross reactivity to other Neisseria.

Schaefer, Keller and Doyle (42) have shown that wheat germ lectin agglutinated 164/165 strains of N. gonorrhoeae and only 1/23 N. meningitidis. However, further studies have shown that non-encapsulated N. meningitidis strains are also agglutinated while the same strains with intact capsules are not agglutinated (43). This suggests that the polysaccharide capsule prevents interaction of the lectin and the LPS. Wheat germ lectin is specific for N-acetyl-D-glucosamine, a component sugar of gonococcal and some meningococcal lipopolysaccharides.

Enzyme-Linked Immunosorbent Assay

In 1966 Nakane and Pierce (44) described the use of horse radish peroxidase labelled antibody for the detection of yolk sac carcinoma in tissue sections. They noted that the method was better than the fluorescent antibody technique due to the permanence of the reaction and the fact that an ordinary light microscope could be used. The conjugate could penetrate tissue as well as the antibody alone. They commented that the ability of the conjugate to amplify the reaction at the site of the antigen would prove useful in biological research.

In 1971 Van Weeman and Schuur (45), and Engvall and Perlman (46) independently published the details of enzyme immunoassays for which the latter group derived the name ELISA or Enzyme Linked Immunosorbent Assay.

The basic principle of the test is easily understood. In the direct test, antigen is absorbed or bound to a solid support, depending on its inherent nature. Any unbound antigen is then washed from the support. Antibody specific for the antigen is added to the system and incubated, unbound antibody is then washed away. Anti-immunoglobulin which has previously been bound to an enzyme, usually either horse radish peroxidase or alkaline phosphatase, is incubated with the antigen-antibody complex. The unbound conjugate is washed from the complex and a substrate is added. The substrate, which is colorless, then

reacts with the enzyme to give a color reaction. The amount of color that develops is proportional to the amount of antibody which has attached to the antigen.

For detection of antigen a double sandwich technique can be used. A coating of antibody is absorbed to a solid support and then nonabsorbed antibody is rinsed off. Antigen, of unknown quantity, is incubated with the support and unbound antigen is then washed from the support. A second antibody, from another species of animal, but specific for the same antigen is incubated with the support, then the unbound portion is washed off. An anti-immunoglobulin conjugate specific for the second species antibody is added. After a further incubation and washing step, substrate is added which will give a color development dependent on the amount of antigen present.

A competitive assay for antigen detection may also be done. Antibody is absorbed to the carrier surface. A known amount of antigen which has been conjugated to an enzyme label is added to the washed carrier. At the same time an unknown amount of antigen is added to a duplicate carrier in the presence of the same amount of labelled antigen. Following incubation and washing substrate is reacted with the carrier-antibody-antigen complex. The known positive control will have maximal color development. The well containing unknown amounts of antigen will have variable color developed dependent on the amount of antigen present. The less intense the color the more unlabelled antigen is present.

These are just a few of the many variations on enzyme linked immunosorbent assays which may be performed.

Solid phases used in ELISA tests have included polyvinyl, polystyrene, cellulose, cross-linked dextrans, polyacrylamide, glass or silicone rubber in shapes of tubes, microtiter, plates, discs or beads (47). The microtiter plate systems tend to outnumber the others due to ease of handling, washing and the number of samples which may be handled in a limited space.

Lentonen and Viljanen (48) compared polystyrene tubes, nylon rings and cyanogen bromide activated paper as carriers. They bound radioactive bovine serum albumin (BSA) to the various carriers and performed radioactive counts after each step in the ELISA procedure. It was found that it took approximately six washes to rinse away unbound BSA. Antigen detachment ranged from 60% for nylon, 25-37% for polystyrene (dependent on batch variation) and approximately 13% for the activated paper. Zollinger et al. (49) reported a 6-8% leakage rate using a meningococcal antigen coating on polyvinyl. Salomen and Vaheri (50) reported a total adherence of approximately 21% for IgG, 78% for IgM and 12% for IgA to polystyrene in a radioactive trace study. They further noted that the maximal amount of protein absorbed to the support greatly exceeded the amount of protein needed for maximal reactivity of the test.

For most purposes coating of a protein antigen at an alkaline pH for a few hours seems to be a sufficient means of coating the support. The fixation of antigens to the support may require various fixation processes. Capsular

polysaccharide has been cyanogen bromide coupled to poly-L-lysine, which is then passively absorbed to the support (51). Virus particles have been acetone fixed (52) or crosslinked to a fetal calf serum precoating with glutaraldehyde (53).

The purer the antigen preparation the better the test, as there is less competition for binding to the support. The use of Tween 20 (47) or other wetting agents in the wash buffer decreases the uptake of non-specific material on the support. In most cases the sensitized solid phase is stable for a period of several months.

The enzyme used in conjugation should preferably have the following characteristics:

1. Highly purified at low cost.
2. Residues through which it may be linked to antibody/antigen and retain enzyme activity.
3. High specific activity.
4. Stable.
5. Similar enzyme activity absent in the system to be detected.
6. Sensitive, inexpensive assay method.
7. Minimal health hazard.

The most commonly used enzymes are peroxidase, β - galactosidase and alkaline phosphatase. These may be conjugated by a number of methods, the most common being the one step glutaraldehyde method. This involves the mixing of the enzyme and antibody in the presence of glutaraldehyde and

subsequent separation by chromatography of the conjugated and unconjugated material. This is the most common method of producing alkaline phosphatase conjugates. While this is a simple method it results in the loss of antibody and enzyme activity due to the complexing of the like molecules (54).

A two step glutaraldehyde method can be used to conjugate peroxidase. Enzymic activity of peroxidase is not decreased by glutaraldehyde, unlike other enzymes. The peroxidase is activated with glutaraldehyde, then chromatographed free of the excess. The activated enzyme is then reacted with antibody. This method usually gives a superior product compared to the one step method (55).

Glycoproteins such as peroxidase may be coupled by means of periodate oxidation. Amino residues on the enzyme are blocked with fluorodinitrobenzene and the enzyme oxidized with periodate. Free amino groups in the antibody are bound to the aldehyde groups formed on the enzyme by the periodate oxidation. Conjugation of up to 70% of the enzyme and 99% of the antibody has been reported (56).

Depending on the enzyme to be linked various other methods may be used (57).

The substrate is usually chosen so that on initial application it is colorless and with development becomes colored. The reaction may be read visually or spectrophotometrically. Either a soluble or precipitating substrate may be used depending on the type of test performed.

For histopathological studies a light dense precipitating stain would be necessary, while for a spectrophotometric test a soluble one would be best. For peroxidase there is a list of approximately 20 substrates of varying characteristics which may be used (57). Some, however, are carcinogenic and are not recommended.

To date approximately 50 infectious diseases have been diagnosed using ELISA techniques. Measles and hepatitis kits are presently being marketed. Millipore^{RT} markets enzyme tests for Entamoeba histolytica and Toxoplasma antibody. It is extensively used in assays for hormones (58).

In comparison to radio immuno assays, ELISA has the same sensitivity, specificity and reproducibility. ELISA test kits have a longer shelf life and lower cost per test that does RIA. If the substrate is carefully chosen the ELISA has a lower health risk than does the RIA.

METHODS

Organisms and Culture Media

The origin of the strains of Neisseria gonorrhoeae Gc 6 and G 9 colony type 4 has been described (15). The Gc 6 strain was obtained from the Public Health Laboratory, Ottawa, Ont.. G 9 was obtained from Dr. Herman Schneider of the Walter Reed Army Institute, Washington, D.C. The Neisseria meningitidis strains used were the standard serogroup reference strains and were obtained along with the gonococcal strains from the National Neisseria Reference Center, Ottawa, Ont. courtesy of Dr. Fraser Ashton.

The bacteria were maintained in frozen culture in trypticase soy broth containing 20% glycerol. Seed cultures were grown on GC agar (Difco) containing 2% defined supplement (5) and 0.002 % yeast extract, for 16 to 20 hr at 36° C. in a 5% CO_2 atmosphere. The cells were suspended in Dulbecco's phosphate buffered saline (pH 7.2) and then seeded onto GC agar in 50 X 150 mm disposable Petri plates. The plates were incubated as for the seed plates. Growth was harvested by scraping the agar surface with a rubber spatula, and was frozen at -70° C until used.

Extraction of Lipopolysaccharide

LPS was extracted by an adaptation of the method of Johnson and Perry (59). Bacteria (500 g) were placed in

500 ml of 50 mM sodium phosphate buffer (pH 7.2) with 5mM ethylenediaminetetraacetic acid (EDTA), hen egg white lysozyme (0.5 g, Sigma grade 1, 25000 units/mg) was added and the mixture incubated overnight at 37° C. The suspension was adjusted to 10 mM with respect to $MgCl_2$. Ribonuclease (2500 U/mg, Sigma) and deoxyribonuclease (1400 U/mg, Sigma) were added at a final concentration of 0.5 µg/ml. This suspension was incubated at 37° C for 1 hr.

The cell lysate was mixed with a Sorvall omnimixer at top speed until warm to the touch, approximately 5 minutes. An equal volume (500 ml) of 90% phenol which had been heated to 65° C was then added. The suspension was maintained at 65° C for 20 minutes while being stirred. The mixture was cooled to 10° C while being stirred in an ice bath. Centrifugation at 10,000 x g for 20 min. separated the mixture into aqueous, interfacial and phenol phases.

The aqueous phase was aspirated and retained. The interphase was removed by aspiration and made up to its original volume (500 ml) with distilled water. The phenol phase was poured off and re-heated to 65° C. The interphase was mixed and re-extracted with the phenol as before. This process was repeated a third time. The pooled aqueous layers were dialized against running tap water for four days and then lyophilized.

This crude LPS was suspended in distilled water at a

concentration of 27 mg/ml and centrifuged at 1000 x g for 5 minutes. The sediment was discarded and the supernatant fraction was centrifuged at 105,000 x g for 16 hr in a Beckman ultracentrifuge. The purity and content of LPS in the fractions were assayed by the carbocyanine dye assay (59). Sequential ultracentrifugations were carried out until the preparation was considered pure. LPS extracts of other Neisseria species, prepared in a similar manner were obtained from Dr. M.B. Perry, National Research Council of Canada. Escherichia coli LPS was obtained from Difco.

Carbocyanine Dye Assay

Five mg of carbocyanine (1-Ethyl-2-[3-(1-ethylnaphtho [1,2-d] -thiazolin-2-ylidene)-2-methylpropenyl]naphtho [1,2-d] thiazolium Bromide, Eastman Kodak Co.) was dissolved in 10 ml of 1:1 sodium acetate buffer (0.03 M, pH 4.0):1,4 dioxane mixture. To this were added 40 ml of 0.03 M sodium acetate buffer for a total of 50 ml of dye reagent. The test solution consists of 1.0 ml (0.01 %)LPS, 0.4 ml sodium acetate buffer and 0.6 ml dye reagent. An absorbance spectrum is run from 700 to 350 nm (59).

The reaction of LPS with the carbocyanine dye reagent produces a spectral shift from a maximal absorption of 510 nm to 460 nm; nucleic acid produces adsorption at 550 nm and polysaccharide at 600 nm. LPS was considered pure if a single peak of adsorption at 460 nm was obtained.

Competitive Inhibition ELISA

The method of Ito et al. (63) was used for coating the microtiter plate wells. Purified LPS (100 µl, 20 µg/ml in 0.01 M carbonate buffer pH 9.5) was added to microtiter plates (E.I.A. Linbro). To this was added 0.02 M $MgCl_2$ (100 µl). The plates were incubated for two hrs at 37° C and then washed with 0.02 M $MgCl_2$. Various saccharides and disaccharides were diluted out directly in the plates in ELISA buffer (0.01 M PBS, pH 7.6 with 0.01 % Tween 20, 0.01 % bovine serum albumin and 0.002 M $MgCl_2$). All assays were done in triplicate. To the 100 µl of sugar were added 100 µl of predetermined antibody dilution (by box titration). Control wells without inhibitors were also included. The plate was incubated for 1 hr at 37° C and then washed with ELISA buffer three times. Anti-immunoglobulin conjugated to horse radish peroxidase (Zymed) at a predetermined dilution (by box titration) was added to the wells and incubated for 1 hr at 37° C. The plate was washed again three times with ELISA buffer. Substrate (200 µl, O-phenylenediamine 8 mg, 30 % H_2O_2 5 µl, in 20 ml citrate buffer pH 5.6) was added and incubated for 1 hour at room temperature in the dark. The plates were read on an ELISA reader (Flow Laboratories, Multiscan) at 450 nm.

All sugars used were obtained from Pfanstiehl Laboratories, Inc.

Radioiodation of Lipopolysaccharide

Methyl para-hydroxybenzimidate HCL (MPHBIM) was prepared from parahydroxy benzonitrile (Eastman Kodak Co.) by the method of Wood et al (60). MPHBIM 10 ml of 50 mM solution in 0.05 M borate buffer (pH 8.0) was added to the purified LPS (10 mg) and incubated for 18 hrs at 37° C. The resultant product, M-LPS, was dialysed versus 4 1 L changes of PBS at 4° C.

A modified method of Ulevitch (61) was used to radiolabel the M-LPS. To the 10 ml of M-LPS (1 mg/ml) were added 5 µl of 0.1 N HCl, 50 µl of 1 % -D glucose, 100 µl Enzymobeads (Bio Rad Laboratories) and 5 mCi $Na^{125}I$ (New England Nuclear, carrier free). This suspension was mixed for 30 min. at room temperature. The reaction was stopped with 50 µl of 0.01 % NaN_3 followed by centrifugation. The supernatant was suctioned off the Enzymobeads and dialysed versus 6 changes of PBS (pH 7.2) at 4° C over a 4 day period.

Antiserum and Monoclonal Antibodies

The capon anti-gonococcal LPS antiserum used was produced by the method of Diena et al. (62). A group of 20 caponized Leghorn roosters, obtained from King Capon Ltd., P.O. Box 150, Oak Ridges, Ont., were maintained on an antibiotic free diet. Capons were immunized intravenously via the medial wing vein once a week for 5 weeks. All injections were 0.5 ml of a 500 ug/ml solution of LPS in sterile distilled water. The capons were bled by cardiac puncture 1 week after the final injection. The sera were pooled and called lot 19. Serum produced in a similar manner provides the basis for a slide

agglutination test for N. gonorrhoeae and is distributed by O.C.W. Diagnostics Ltd., Rexdale, Ont.

The hybridoma cell lines were supplied by Dr. T. Pearson, University of Victoria, B.C. Cell line C1 6 1/40.2.30 was obtained from a fusion of spleen cells of a mouse immunized with purified Gc 6 LPS. It is an IgM with the ability to bind to staphylococcal protein A.

The other two clones were from a fusion of spleen cells from a G9 LPS immunized mouse. They are both IgG producers and are designated G9 1/23.17.6 and G9 1/26.23.11. Two monoclonal antibody clones specific for Chlamydia trachomatis; Chl 1/29.14.29 and Chl 2/5 8-10, also obtained from Dr. T. Pearson were tested as controls.

Monoclonal immunoglobulin typing was done in the laboratory of Dr. T. Pearson by means of a radioimmunoassay.

Ascites fluid was produced in pristane (2,6,10,14-Tetramethylpentadecane, Aldrich Chemicals) primed mice. Each mouse was injected with 10^5 hybridoma cells in 0.5 ml of RPMI 40 tissue culture fluid into the sterile abcess formed by the pristane, one week after the pristane injection. All mice developed palpable tumors by 7 to 10 days. Fluid was removed by piercing the tumor with an 18 gauge needle and allowing the fluid to drip into a test tube. This procedure was repeated 2 more times at 3-4 day intervals.

The ascites from each tapping was pooled according to cell type and clotted overnight at 4° C after centrifugation at 2,000 x G to remove cell material. Unclotted fluid was suctioned from the clot and centrifuged at 10,000 x G for

20 minutes to remove particulate matter. Ascites fluid was stored in 1 ml aliquots at - 20° C. After thawing at 37° C the fluid was centrifuged at 10,000 x G to remove any further particulate matter.

Immunoprecipitation and Radioimmunoassay.

Immunoprecipitation inhibition tests were performed by the method of Kabat (64). To 50 ul of immune capon serum was added a 200 µl dilution of saccharide plus 50 µl of 0.02 M $MgCl_2$. All immunoprecipitation dilutions were done in PBS (0.01 M with 8 % NaCl, PBS8). The tubes were mixed for 1 hr at room temperature. LPS (100 µg) was added in 200 µl of PBS8. This had previously been determined to give optimal precipitation. To this were added 500 µl of PBS8 to bring the total volume to 1 ml. The tubes were left at 4° C for four days. The tubes were then centrifuged, the supernatant aspirated and the contents washed with PBS8. After re-centrifugation the process was repeated two times.

The precipitate was suspended in 0.1 ml of distilled water and assayed for content of protein by the Bio Rad protein assay method. Briefly, 5.0 ml of diluted dye reagent was added to the tube and vortexed. The absorbance was read 5 minutes later at 595 nm. Protein content was determined by comparison to a standard curve of bovine gamma globulin.

Radioimmunoassay (RIA) inhibition tests were performed with monoclonal antibodies by the following method. Ascites fluid (2 ml) containing monoclonal antibody was mixed with 10 ml of a 10 % suspension of Sepharose Protein A (Pharmacia Fine Chemicals) for two hours at room temperature. PBS (0.01 M, pH 8.5) was used throughout this assay. The Sepharose beads were pelletted by low speed centrifugation and washed three times with PBS. These beads (Seph-Ab) were made up to the original 10 % suspension in PBS. Seph-Ab (50 µl) was incubated with dilutions of inhibitor (100 µl) for 1 hour at room temperature. Radioactive LPS solution (25 ug in 50 µl) was added to the Seph-Ab suspension and mixed on a rotary shaker for 2 hours at room temperature. The beads were centrifuged and the supernatant suctioned off. The beads were washed three times with PBS and then counted in a gamma counter (Nuclear, Automatic).

Analysis of the Data

Inhibition of the ELISA, RIA and immunoprecipitation methods was analysed by linear regression analysis. The computer program for the analysis was provided by Lise Bramalle of the Biostatistics Division of the National Research Council of Canada.

The sigmoidal inhibition curves obtained from the raw data were transformed by computer to absorbance versus $\log_{10}$ concentration of inhibitor to provide a linear plot. Linear

regression analysis was performed on the resultant data and the slopes of the inhibited reactions were compared to the non-inhibited standards, which have a slope of zero. Sugars or LPS's which produced a significantly different slope, as determined by the Student T test, were considered to have inhibited the reaction. In certain cases in which strong inhibition was evident, although the slope was not significantly different from zero a Student T test was performed on the Y intercept.

Percent inhibition graphs were plotted using the non-inhibited reaction as zero percent inhibition and determining the percent reduction in absorbance caused by the inhibitor. Percent reduction was plotted versus $\log_{10}$ concentration of inhibitor.

Results

The yield of purified LPS from 500 grams (wet weight) of Gc 6 cells was 4.68 grams or 0.936 %. The G 9 cells, 230 g, yielded 2.65 grams or 1.15 % LPS. This is in good agreement with the 1 to 1.7 % found by Perry et al. (5).

The carbocyanine dye assay results of the Gc 6 purification (fig 1) show that the LPS is pure. The nucleic acid (550 nm) and polysaccharide (600 nm) peaks are not evident in the final preparation. The unpurified crude LPS tracing demonstrates the masking effect that the polysaccharide and nucleic acid have on the LPS peak.

The LPS contained 0.01 % protein as determined by the Bio Rad protein assay, completed as directed on the package insert for the micro test, using bovine gamma globulin as a reference standard.

Kabat (64) has reported that a concentration of 8% NaCl is necessary for the formation of immunoprecipitation complexes when using fowl serum. For this reason its effects on the CI-ELISA were studied (Fig. 2). A concentration of 8% NaCl was used in the ELISA buffer for the incubation of the capon antibody and lactose in the LPS coated microtiter plates. All subsequent steps were completed with 0.85% NaCl ELISA buffer.

Less inhibition was observed at the 8% salt concentration than at the 0.85% NaCl concentration. All subsequent experiments were completed in 0.85% saline.

The effect of antiserum dilution on the inhibition by lactose is shown in figure 3. The greater the dilution the more inhibition is evident. For comparison to the precipitin inhibition test an antibody dilution of 1:400 was chosen.

Lactose (4-O(β-D galactopyranosyl)-D-glucopyranose) a component sugar of gonococcal LPS (5) and its structural analogues (3-O(β-D(galactopyranosyl)-D-arabinopyranose (galactose-arabinose) and melibiose (6-O(α-D(galactopyranosyl-D-glucopyranose) all give significant inhibition in the precipitin reaction, as does D-galactose itself (fig. 4). The β-D galactopyranosyl structure is involved in the antibody reaction as evidenced by two facts: first, the galactose-arabinose, which contains a β-D galactopyranose linkage, is a better inhibitor than is melibiose which contains a α-D linkage and second, galactose alone inhibits the reaction, whereas, glucose does not. Maltose, (4-O(β-D-glucopyranosyl)-D-glucopyranose) does not inhibit the precipitin reaction. Arabinose, sucrose, and N-acetyl-D-glucosamine have no inhibitory effect.

Similar results were obtained in the CI-ELISA (fig. 5). Glucose, ribose, N-acetyl-mannosamine, glucosamine, galactosamine, arabinose, cellobiose, maltose, and N-acetyl-D-glucosamine have no inhibitory effect in either the CI-ELISA or the precipitin inhibition test.

At a higher dilution of serum, 1:800 as opposed to 1:400, N-acetyl- D-glucosamine is inhibitory in the ELISA test system (fig. 6). No significant inhibition is evidenced by this sugar in the precipitin inhibition test at the optimal concentration of antigen/antibody. N-acetyl-D-glucosamine is a component sugar of the gonococcal core as determined by sugar analysis (5) and lectin studies (41). The inhibitory effect of lactose and galactose are also increased. Glucose, ribose, arabinose, and cellobiose had no inhibitory effect at the higher dilution.

The inhibition caused by gonococcal LPS and its de-acetylated and core derivatives is shown in figure 7. The polysaccharide core is the best inhibitor of the ELISA reaction.

The inhibition caused by LPSs of other Neisseria species is demonstrated in figure 8. Neisseria mucosa, Neisseria meningitidis and Neisseria lactamica LPS all of which contain a lactose determinant (31) are inhibitory to the capon antiserum. Neisseria ovis, and Neisseria denitrificans do not contain lactose (32) and have no inhibitory effect. The unrelated LPS

of E. coli 055:B5 and 011:B4 had no inhibitory effect.

Monoclonal mouse ascites fluid, like polyclonal capon antiserum, was inhibited by lactose to a greater extent at higher dilutions of ascites fluid (fig. 9). A 1:6400 dilution of ascites fluid was used in subsequent experiments.

The results of the precipitin test performed with the radioactively labelled LPS and monoclonal antibody Cl 6 1/40.2.30 bound to Sepharose-Protein A are shown in fig. 10. Only lactose had an inhibitory effect. Other sugars tested were: glucose, galactose, galactose-arabinose, sucrose, arabinose, ribose, maltose, N-acetyl-D-glucosamine and KDO.

The results of the RIA inhibition test agree with those of the CI-ELISA performed using the monoclonal ascites fluid (fig. 11). The RIA was performed in a pH 8.5 buffer to ensure the adherance of the monoclonal IgM to the Sepharose-Protein A (personal communication T. Pearson, 66). N-acetyl-mannosamine and galactosamine were tested in the CI-ELISA in addition to the sugars noted for the RIA.

Also included in figure 11 are the results of inhibition with gonococcal LPS and core. The homologous Gc6 core was a better inhibitor than the LPS, as was noted for the capon antiserum. Heterologous gonococcal LPS IN 21 (not shown) and G 9, while potent inhibitors at high concentrations, lose their inhibitory effect much faster than does the homologous LPS.

N. lactamica had a slight inhibitory effect at the highest concentration. *N. mucosa*. *N. denitrificans*, *N. meningitidis* serogroups A, B, and *E. coli* serogroups 055:B5 and 011:B4 LPS had no inhibitory effect. This indicates that the monoclonal antibody has a conformational specificity for the lactose determinant of the gonococcal LPS.

Figure 12 shows the inhibitory patterns of the two G 9 monoclonal antibodies. One antibody, G 9 1/26.23.11 like the Gc 6 monoclonal antibody is inhibited only by lactose. The other antibody, G 9 1/23.17.6 is inhibited by lactose and to a lesser extent galactose. As previously noted the disaccharide is a more potent inhibitor than is the monosaccharide.

Table 2 lists the potential inhibitors tested with each monoclonal ascites fluid and the lot 19 capon antiserum. As noted previously all three anti-gonococcal monoclonal antibodies were inhibited by lactose, while one was additionally inhibited by galactose. None of the analogues of lactose (melibiose, galactose-arabinose) had a significant inhibitory effect on the monoclonal antibodies. This suggests a conformational specificity of the monoclonal antibodies tested as compared to the antibody pool of the capon antibodies.

The anti-chlamydial monoclonal antibodies were not inhibited by any of the sugars. These antibodies were used as a negative control to test for non-specific inhibition by any

of the sugars. The anti-chlamydial antibodies are specific for an as yet unidentified trypsin sensitive, periodate resistant determinant of the chlamydia particle (unpublished data). This indicates that the antigen detected by these clones are protein in nature. It also testifies to the specific nature of the saccharide inhibition reaction.

Discussion

The capon antiserum is inhibited to a similar extent in both the CI-ELISA (1:400 dilution) and the precipitin inhibition (1:20 dilution) tests. Lactose and its structural analogues, galactose-arabinose and melobiose all inhibited the ELISA reaction. Although melibiose was not tested in the precipitin reactions reported in this paper, Perry et al. (7) found this sugar to inhibit the precipitation of hen antiserum directed to gonococcal LPS. All three of these sugars contain a galactopyranose linkage, lactose and galactose-arabinose in the beta configuration and melibiose in the alpha. The free galactose had a lesser inhibitory effect in the two tests. Taken together these results suggest that the immunodominant feature of the gonococcal LPS molecule is a D-galactopyranosyl $1\xrightarrow{\beta}4$ linkage.

At higher serum dilutions the inhibitory effect of the component sugars is more pronounced. The inhibitor is in greater relative abundance than the LPS and is in a soluble as opposed to a bound state. This would afford a greater opportunity for the antibody molecules to react with the saccharide as opposed to the LPS. The higher the dilution of antibody the greater would be the chance of the antibody contacting a lactose molecule as opposed to an LPS molecule.

Inhibition by lactose is increased from 30 to 75 % and by galactose from 15 to 38 %. N-acetyl-D-glucosamine which had no significant inhibitory effect at the lower dilution of antiserum demonstrates cross reactivity with the antiserum. At the lower dilution the anti-lactose activity, which is predominant may block the observance of N-acetyl-D-glucosamine inhibition.

The polysaccharide core of the LPS cannot be tested in the precipitin reaction as it precipitates antibody by itself (7). In the CI-ELISA the core was shown to be a more potent inhibitor than the LPS. This gives further proof to the contention that it is the core portion of the LPS to which the capon antibody is directed. NaOH treatment de-acylates the LPS molecule, making the molecule more soluble and reducing micelle formation. This renders the core portion more accessible to the antibody, than the native state LPS. These results suggest that the conformation of the lipid portion is not an important factor in the antibody-antigen reaction as this is usually altered on de-acylation.

Many of the other Neisseria have LPSs which crossreact to some extent with the capon antiserum. Jennings et al. (33) noted the similarity of the meningococcal and gonococcal LPSs and have theorized that the capsular polysaccharide of the meningococcus prevents crossreactivity to the antiserum. Further proof of this is the agglutination of non-encapsulated variants of meningococci, compared to the non-agglutination of capsulated strains by wheat germ lectin (43). This is

indicative of the presence of N-acetyl-D-glucosamine free to interact with the lectin in the non-encapsulated strain but not the capsulated. Fluorescein labelled capon anti-gonococcal LPS antibody must be absorbed with meningococci prior to its use in a diagnostic FA test for the confirmation of gonococci (65), to prevent false positive reaction, indicating a sharing of similar antigenic determinants.

The monoclonal antibodies Cl 6 1/40.2.30 and G9 1/26.23.11 were inhibited by lactose, but not any other mono or di-saccharides. The G9 1/23.17.6 was also inhibited by the monosaccharide galactose. None of the three, however, were inhibited by the galactose-arabinose or melibiose analogues, indicating the stringent specificity of the binding sites of each of the three monoclonal antibodies.

The Cl 6 1/40.2.30 monoclonal antibody was inhibited to the greatest extent by its homologous LPS. Other gonococcal lipopolysaccharides inhibited to a lesser degree. Heterologous lipopolysaccharides lose their inhibitory effect more rapidly than does the homologous LPS. This indicates there may be minor structural differences in the gonococcal lipopolysaccharides of various gonococci which are not detected by the polyclonal capon anti-LPS antiserum. N. lactamica's slight inhibitory effect at the highest concentration of LPS might be expected due to the presence of lactose in its structure (31). However, N. meningitidis 604A LPS, which also contains lactose determinants (33) has no effect. This lack of inhibition holds true for the other lipopolysaccharides.

tested. This result suggests an extreme conformational specificity of the antibody for the lactose determinant in the gonococcal core.

Mosmann et al. (67) have reported that pH may have an effect on the specificity of monoclonal antibodies. They found the degree of specificity (crossreactivity) was dependent on the pH of the assay system. The RIA was conducted at pH 8.5 to ensure the binding of the Cl 6 1/40.2.30 IgM antibody to the Sepharose -- protein A (personal communications, T. Pearson, 66). The same results were obtained in the RIA assay as in the CI-ELISA which was completed at pH 7.6, suggesting that this antibody is stable over this pH range, without changing its binding specificity.

Apicella et al. (68) reported on a monoclonal antibody, 3F11, which is directed toward a common locus on the gonococcal LPS. Inhibition studies indicated that lactose, galactosamine and LPS derived polysaccharides of the six gonococcal serotypes inhibit the monoclonal antibody 3F11. It was determined that the active site of the antibody was specific for D-galactosamine-galactose-glucose with the galactose linked $1\underline{\beta}4$ to the glucose. The active site of our monoclonal antibodies may either be smaller than the 3F11 site, have more stringent conformational specificities or recognize different portions or conformation of the molecule, as galactosamine did not inhibit the activity.

A second monoclonal antibody 5B9a described by Morse and Apicella (69) was inhibited by N-acetyl-D-glucosamine and lipid A. It also had a wide range of crossreactivity to other Gram negative bacteria. This may be due to the recognition of the lipid A which is present in a wide range of Gram negative bacteria. N-acetyl-D-glucosamine does inhibit the capon antiserum. These results suggest that N-acetyl-D-glucosamine is a second immunodominant site of the gonococcal LPS which in the small selection of monoclonal antibodies tested here, has not been detected.

The results of the experiments reported here and those of Apicella et al. (68) and Morse et al. (69) confirm and extend the work of Perry et al. (40) in the structural analysis of gonococcal LPS. The three monoclonal antibodies described in this paper and 3F11 demonstrate the immunodominance of the lactose region of the gonococcal core. The 3F11 clone is reactive with all six serotype gonococcal strains. Unreported results from our laboratory suggest that 65 % of clinical isolates can be identified by the Cl 6 1/40.2.30 monoclonal antibody in a direct immunofluorescent assay. The antibodies of the two G9 clones in combination with two further clones not reported on in this work extend the ability to identify gonococcal isolates to practically 100%. This suggests the feasibility of developing a specific diagnostic reagent for the identification of clinical gonococcal isolates.

Summary

This thesis demonstrates the feasibility of using a CI-ELISA method for analysis of antigenic determinants. The results obtained were similar to those of the classical precipitin inhibition test. The CI-ELISA method was a more rapid method and was less cumbersome to perform due to the use of microtiter plates as opposed to test tubes. Less reagent was necessary to perform the CI-ELISA test. The CI-ELISA was a more sensitive test when used with polyclonal capon antiserum. Using the CI-ELISA test additional sugars could be shown to be inhibitory to the antiserum.

The RIA and CI-ELISA tests proved to be of equal sensitivity. However, as noted in the introduction the risk of biological hazard is lower using an ELISA assay.

The D-galactopyranosyl 1β4 glucopyranosyl moiety was proven to be an immunodominant feature of gonococcal LPS. The polyclonal capon serum and individual monoclonal antibody all had most of their activity directed toward this structure. Antibodies developed to this structure may prove useful in the development of diagnostic tests for gonorrhoea.

The CI-ELISA proved to be a rapid, reliable method for the analysis of the active sites of antibody activity. It also proved useful in the structural analysis of complex molecules.

Further proof was provided that the gonococcal core contains antigenic sites common to most gonococci and that it contains in part a D-galactopyranosyl 1β4 glucopyranosyl moiety.

The contributions to knowledge made in this thesis are:

1. The specificity of the immunoprecipitation, RIA and CI-ELISA tests are the same when used to probe the active site of antibody molecules.

2. CI-ELISA is a more sensitive method for the analysis of antibody binding sites than is immunoprecipitation since less reagent volumes are required.

3. Monoclonal mouse antibodies recognize the same major immunodeterminant on the gonococcal lipopolysaccharide that polyclonal chicken antibodies recognize.

The work described reaches the objectives stated for this thesis.

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Composition of Core Oligosaccharides Obtained from LPS Produced by *Neisseria* Species

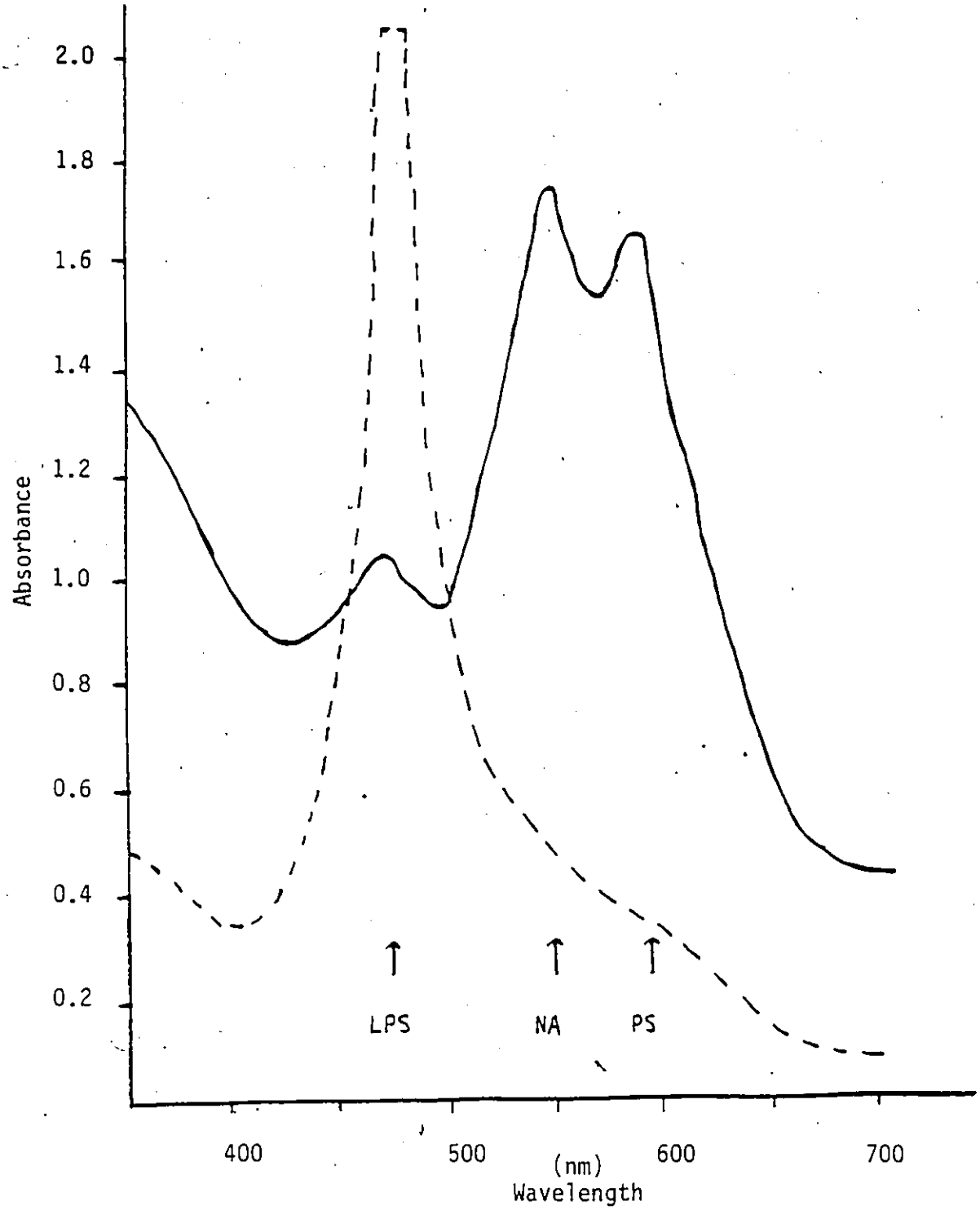
Core Type	Origin	Components KDO	D-Glc	Hep	D-GlcN	L-Rha	D-Gal	EtON	PO4
I	*N. caviae* *N. sicca* *N. cinerea*	1	7	-	-	-	-	-	-
II	*N. cinerea* *N. flava* *N. flavescens* *N. perflava*	1	3	1	2	1	-	1	1
III	*N. flavescens*	1	3	3	-	-	-	-	1
IV	*N. canis* *N. subflava*	1	2	2	-	4-	-	2	1.5
V	*B. catarrhalis*	1	4	-	1	-	1	-	-
	N. gonorrhoeae	1	2	1	2	-	2	-	1
	N. meningitidis	0.9 to 1.3	1 to 2.1	1 to 2	1.4 to 1.9	-	2.4	2	1.6

(1) Moles per core oligosaccharide. KDO = 3-deoxy-D-*manno*-octulosonate; D-Glc = D-glucose; Hep = L-glycero-D-*manno*-heptose; d-GlcN = 2-amino-2-deoxy-D-glucose; L-Rha = L-rhamnose; D-Gal = D-galactose; EtON = ethanolamine.

Table from Perry et al (31) used by permission.

(2) Perry et al (5)
(3) Jennings et al (33)

TABLE 1



LPS lipopolysaccharide
NA nucleic acid
PS polysaccharide

- - - purified LPS
——— crude LPS after initial extraction

Fig. 1: Carbocyanine dye assay of crude and purified Gc 6 lipopolysaccharide indicating the masking effect of nucleic acids and polysaccharides on lipopolysaccharide.

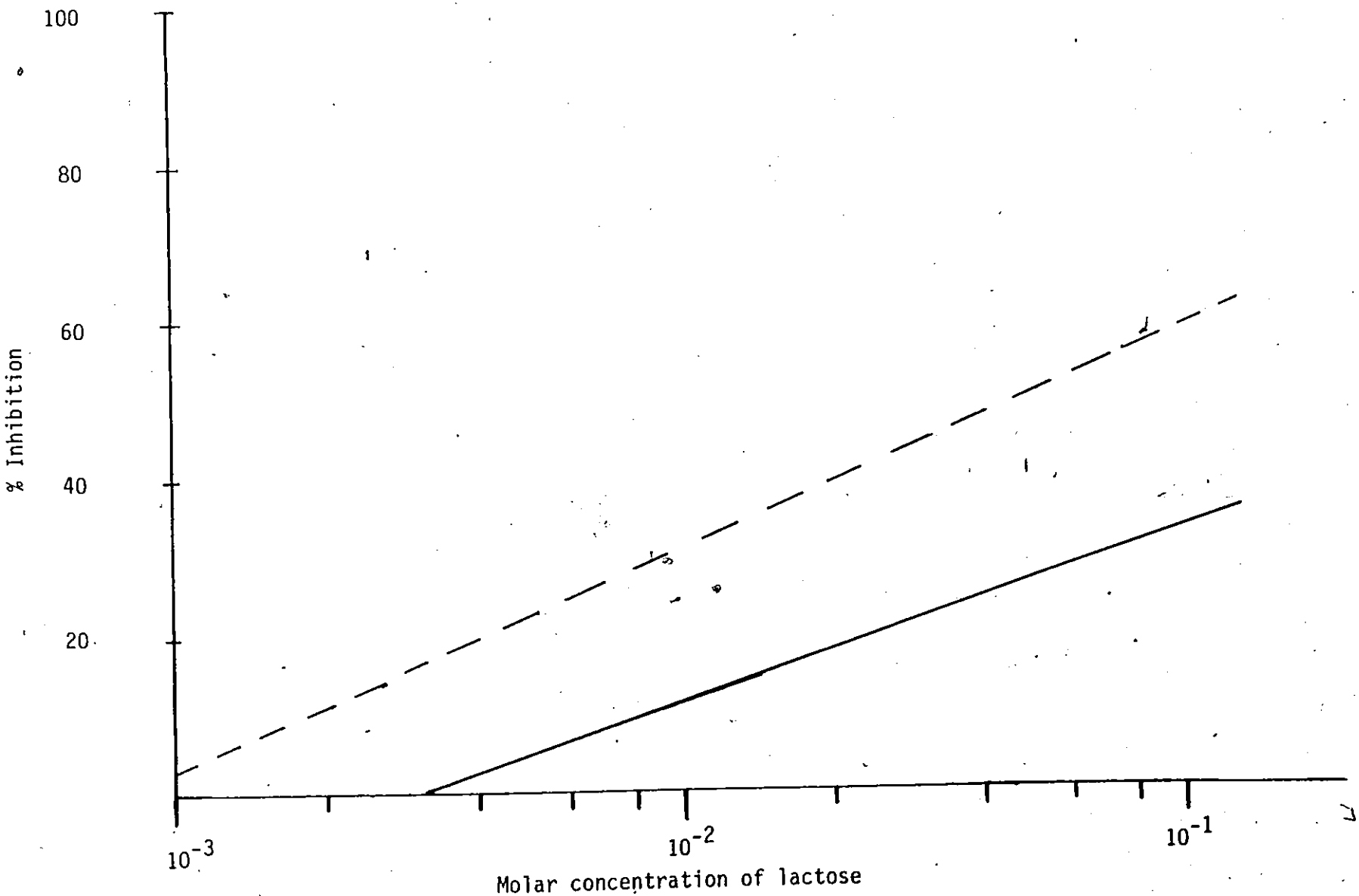


Fig. 2: Effect of NaCl concentration on the inhibition of capon antiserum (1:800) by lactose.

- - - - 0.85% NaCl
——— 8.0 % NaCl

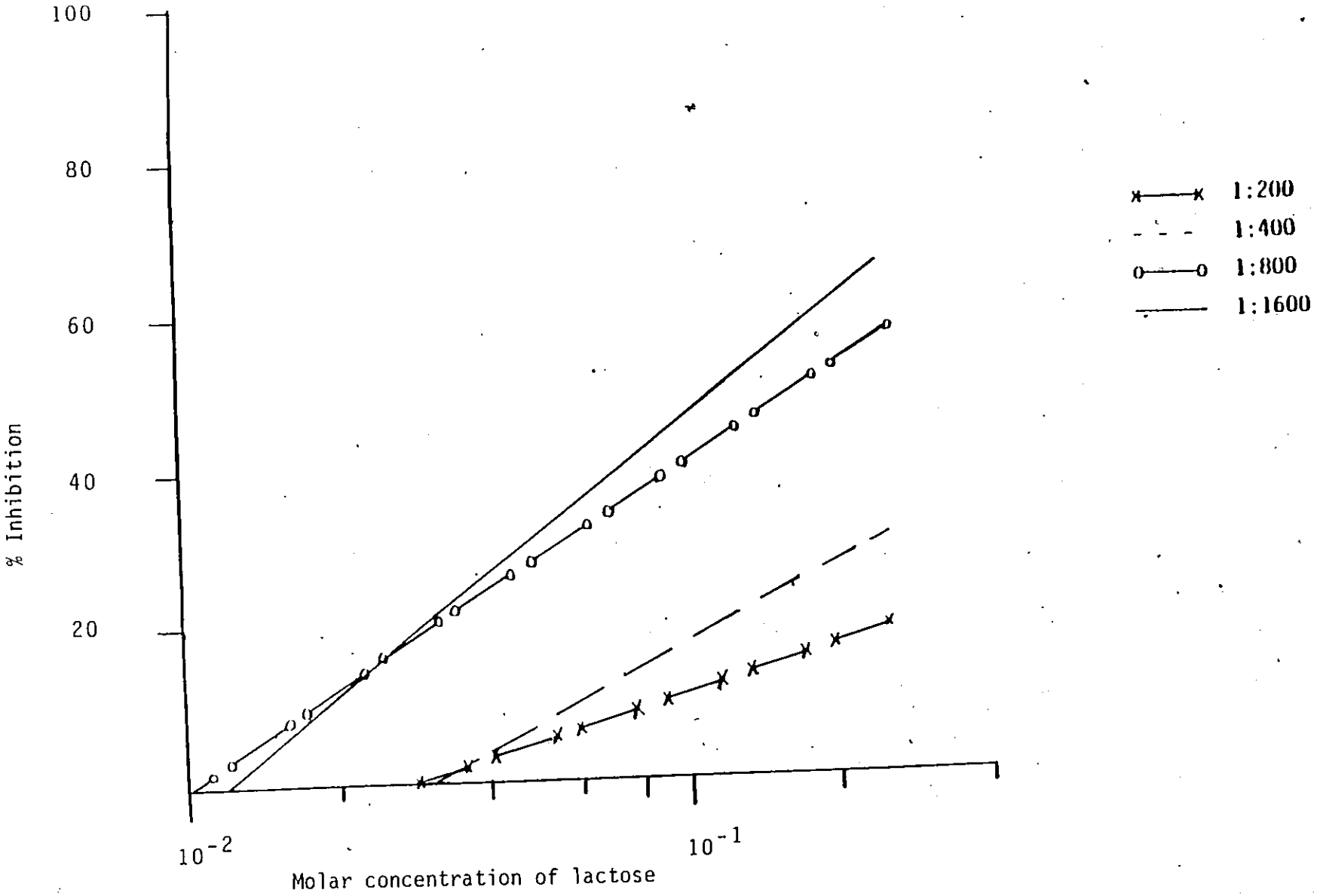


Fig. 3: Lactose inhibition of capon antiserum at various dilutions of antiserum.

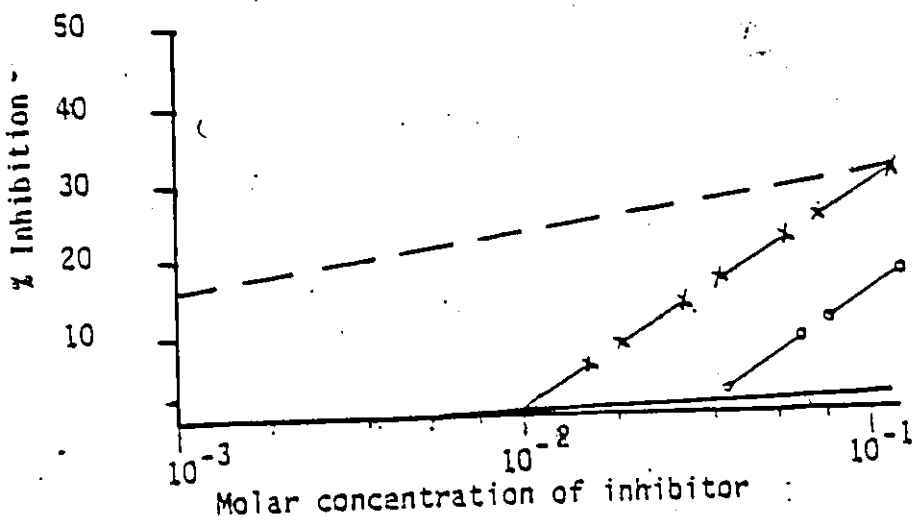


Fig. 4: Precipitin inhibition of capon lot 19 antiserum 1:20 dilution.
S significant inhibition
NS not significant inhibition

- - - - lactose (S)

×——× galactose-arabinose (S)

o——o galactose (S)

——— maltose (NS)

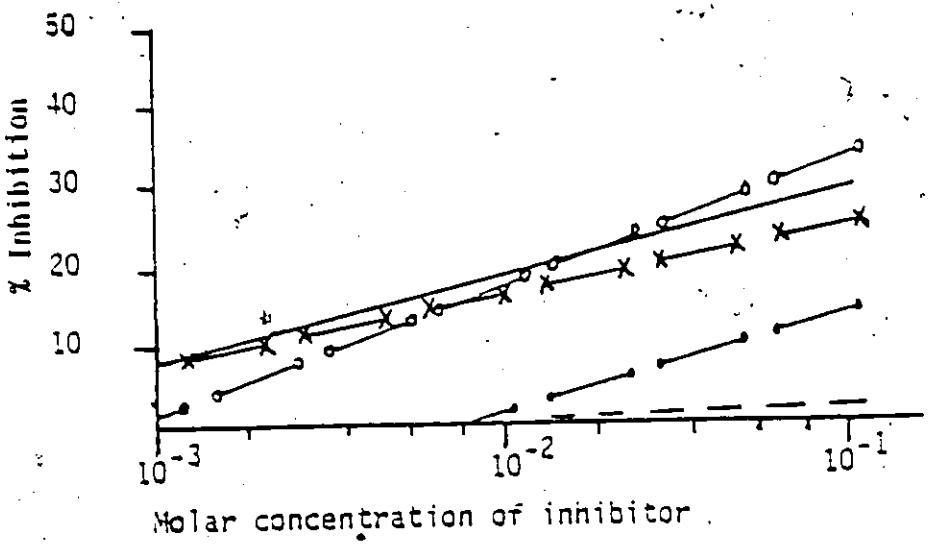


Fig. 5: CI-ELISA. Inhibition results of various saccharides at a 1:400 dilution of capon antiserum.

- - - - maltose (NS)
o——o lactose (S)
——— galactose-arabinose (S)
×——× melobiose (S)
•——• galactose (S)

S significant inhibition

NS not significant inhibition

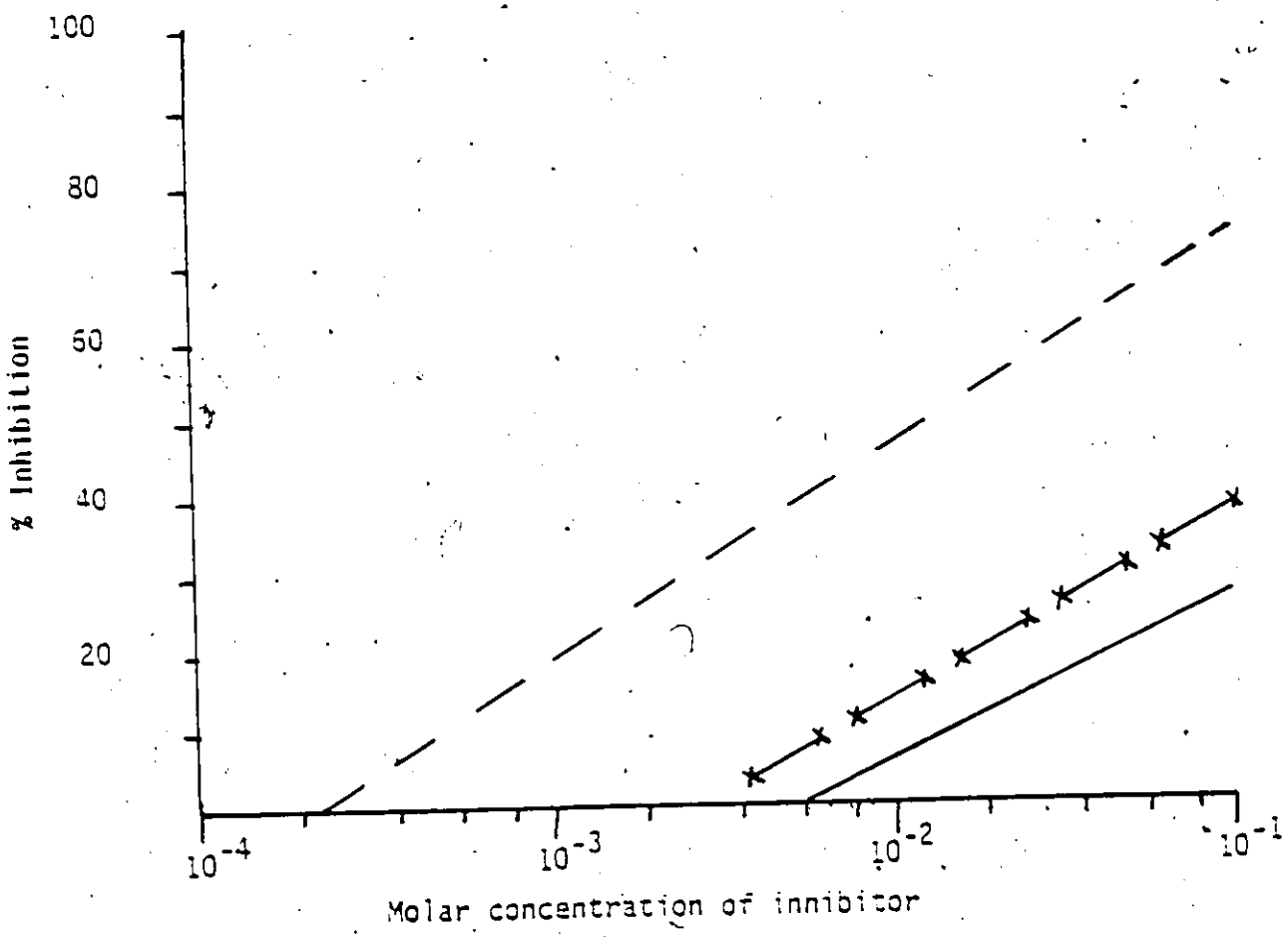


Fig. 6: CI-ELISA. Significant inhibition results of various saccharides at a 1:800 dilution of capon antiserum.

- - - - lactose

×——× galactose

——— N-acetyl-D-glucosamine

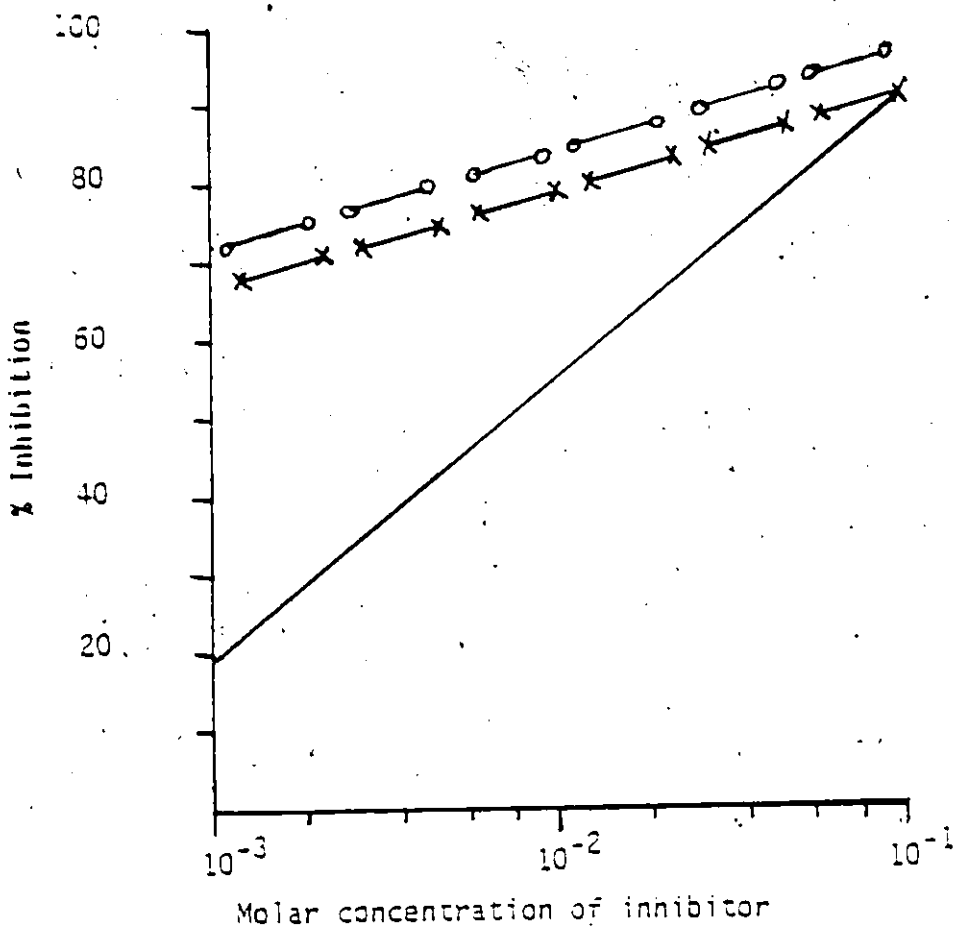


Fig. 7: CI-ELISA. Significant inhibition of capon antiserum (1:400 dilution) by Gc 6 LPS and its derivatives.

——— lipopolysaccharide

o——o polysaccharide core

x——x NaOH deacetylated lipopolysaccharide

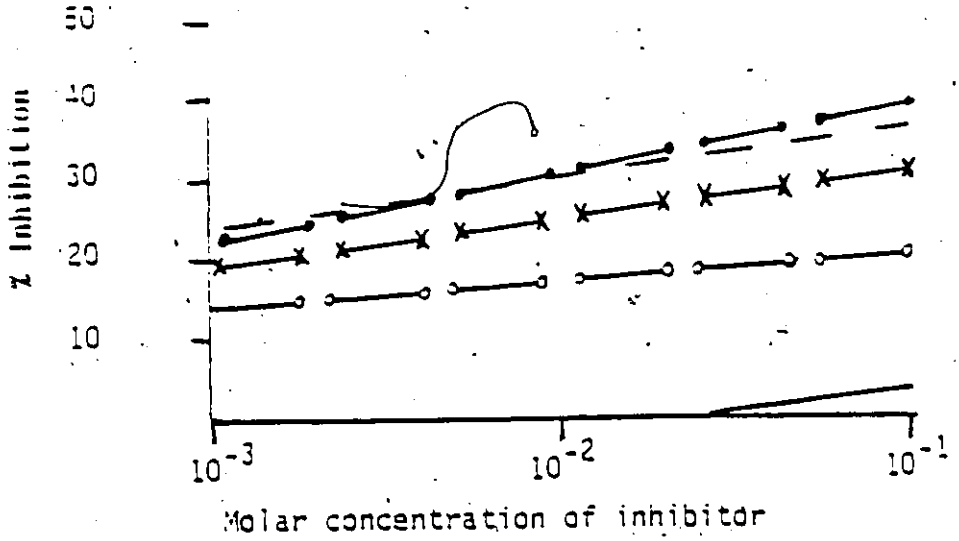


Fig. 8: CI-ELISA. Inhibition of capon antiserum (1:400 dilution) by non-gonococcal LPS.

S significant inhibition
NS not significant inhibition

- N. mucosa (S)
- N. lactamica (S)
- N. meningitidis 604A (S)
- N. meningitidis 6088 (S)
- E. coli 011:84 (NS)

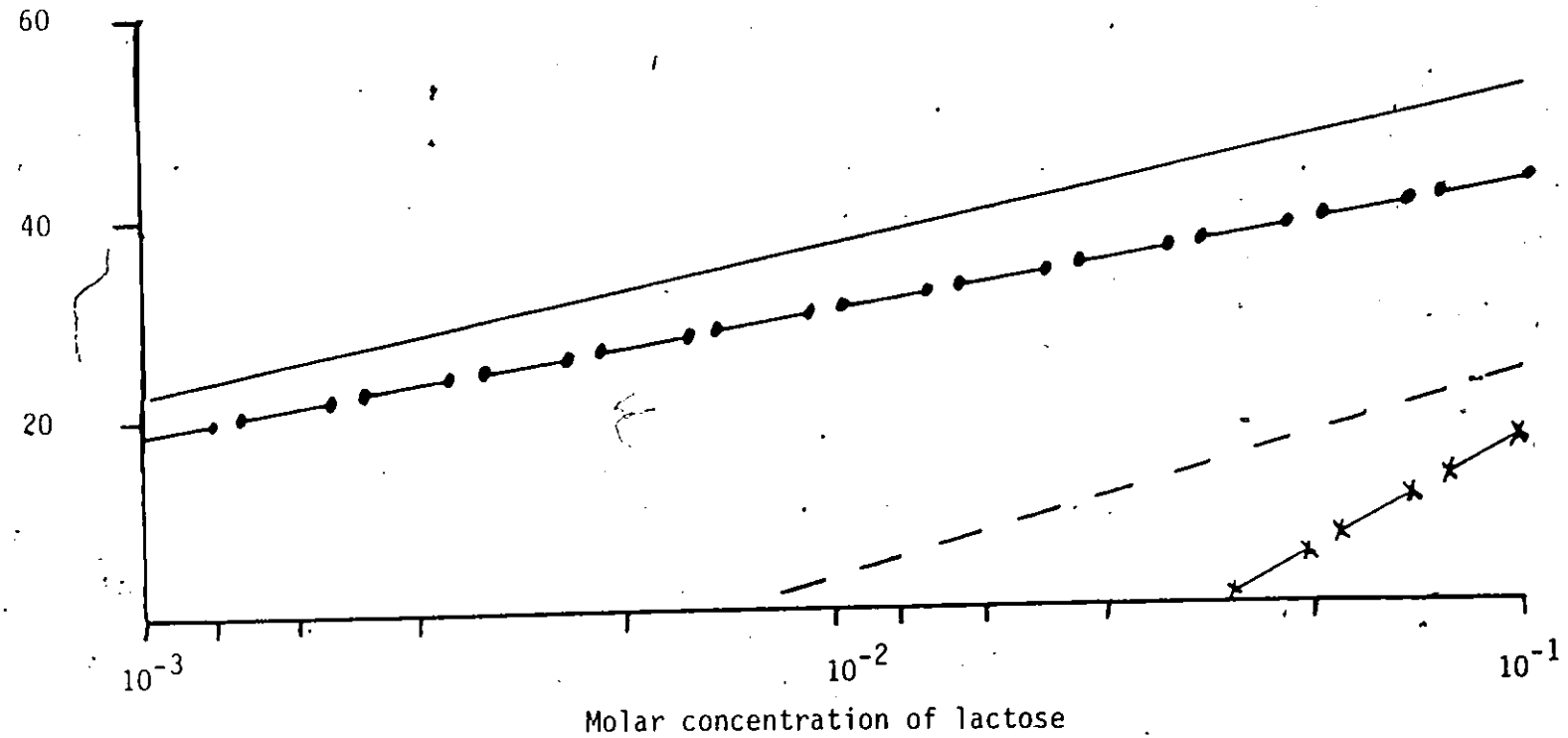


Fig. 9: Inhibition of monoclonal antibody Cl 6 1/40.2.30 by lactose at various dilutions of ascites fluid.

- - - - 1:1600
×——× 1:3200
——— 1:6400
●——● 1:12800

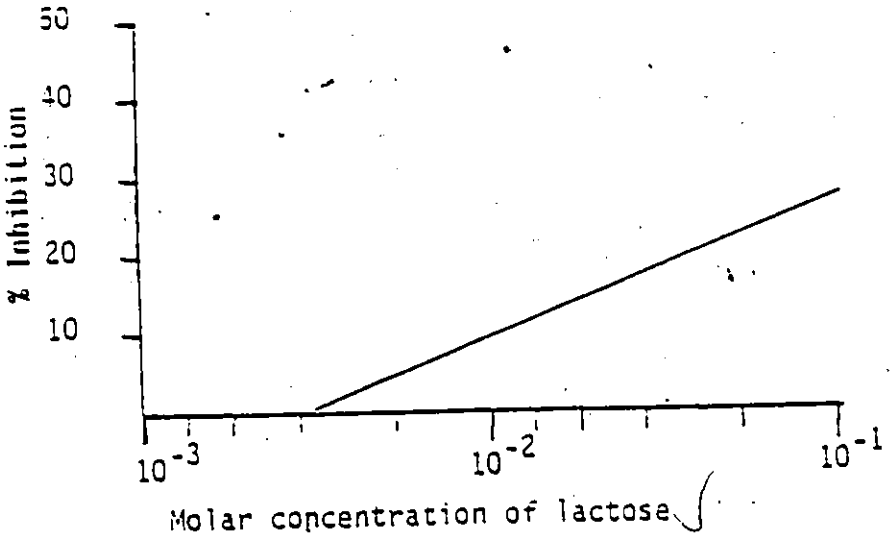


Fig. 10: Inhibition of monoclonal antibody C1 6 1/40.2.30 bound to Sepharose-Protein A detected with radio-active LPS.

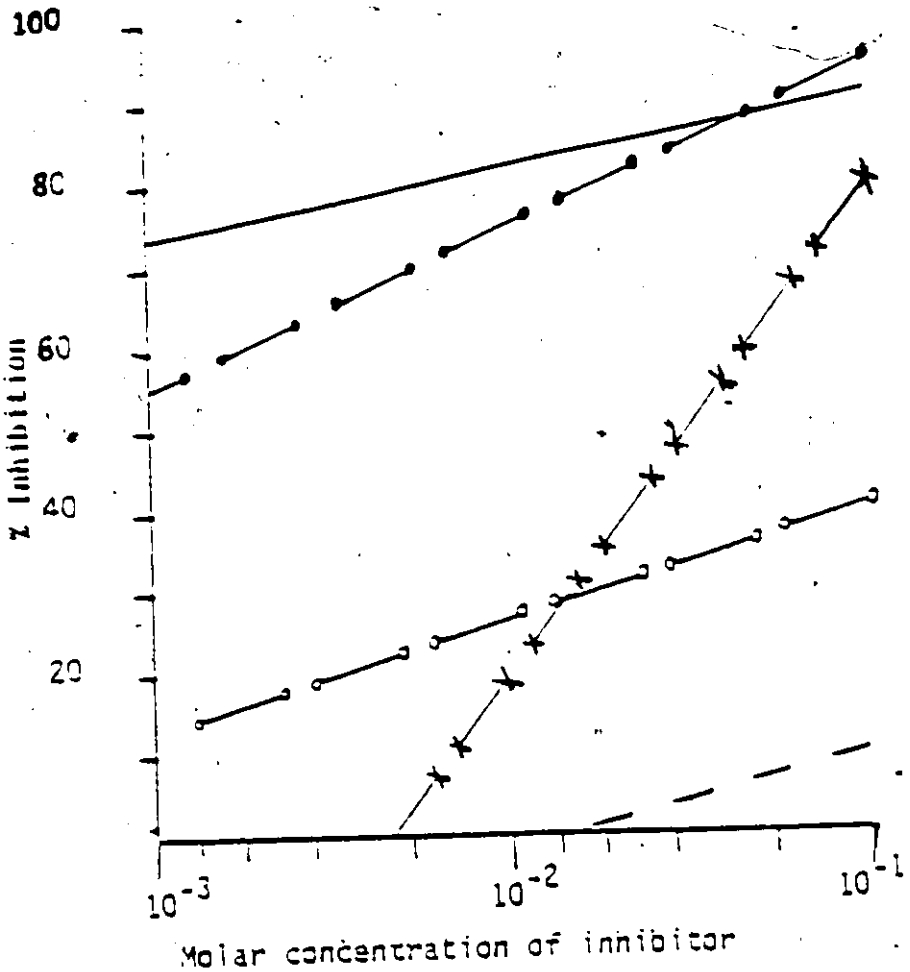


Fig. 11: CI-ELISA. Significant inhibition of monoclonal antibody Cl 6 1/40.2.30 by lactose and lipopolysaccharides.

- Gc 6 lipopolysaccharide
- Gc 6 core polysaccharide
- G9 lipopolysaccharide
- - - - *N. lactamica* lipopolysaccharide
- lactose

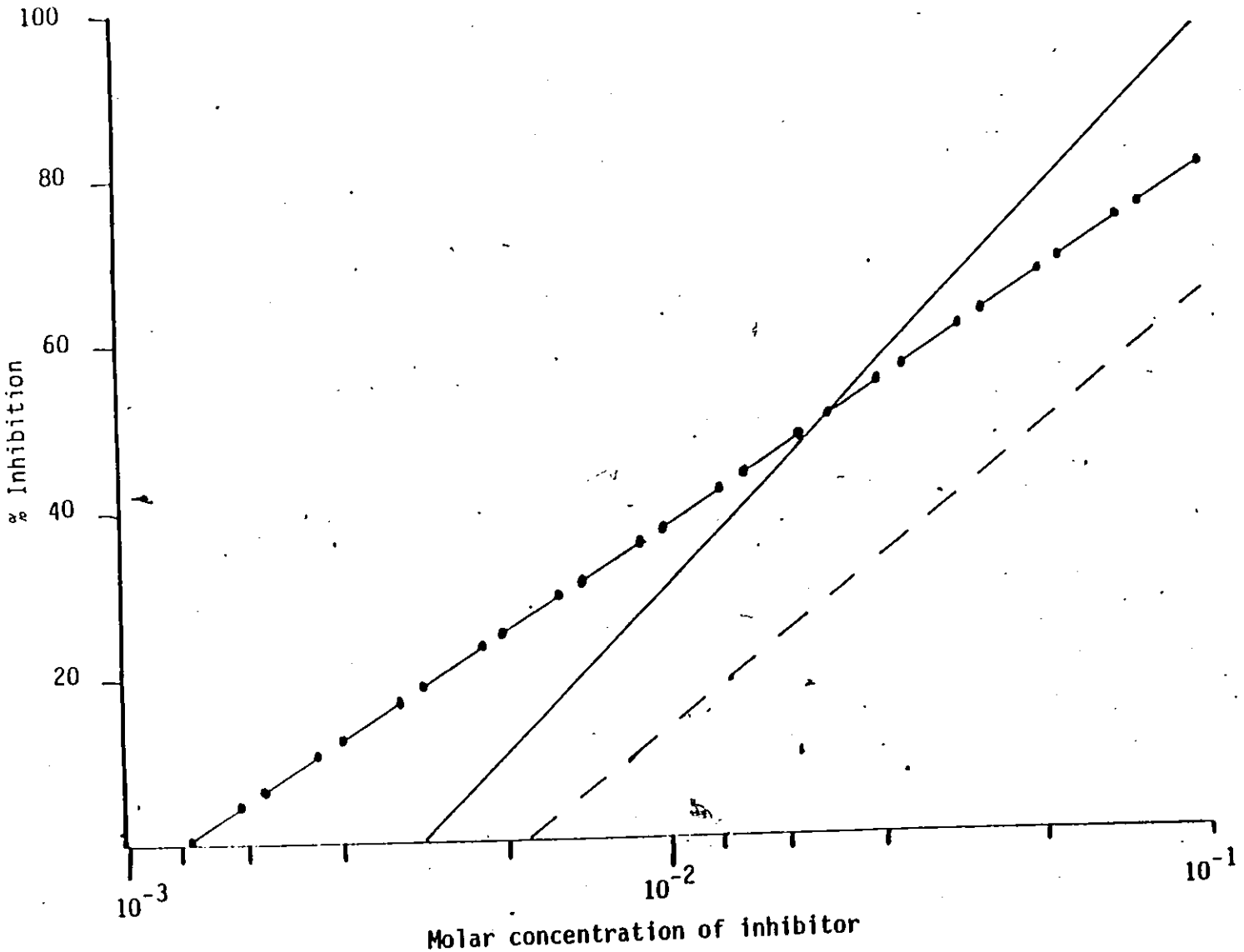


Fig. 12: Significant inhibition of two separate monoclonal antibodies, G9 1/23.17.6 and G9 1/26.23.11.

——— G9 1/23.17.6 lactose

- - - - G9 1/23.17.6 galactose

———● G9 1/26.23.11 lactose

	ANTIBODY TESTED					
INHIBITOR	C16 1/ 40.2.30	G 9 1/ 26.23.11	G 9 1/ 23.17.6	Ch1 1/ 29.14.29	Ch1 2/ 5.8.10	Capon lot 19
Galactose	-	-	+	-	-	+
Glucose	-	-	-	-	-	-
Lactose	+	+	+	-	-	+
Ribose	-	-	-	-	-	-
Maltose	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-
Cellobiose	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-
Galactose-Arabinose	-	-	-	-	-	+
Glucosamine	-	-	-	-	-	-
Galactosamine	-	-	-	-	-	-
N-acetyly-D-glucosamine	-	-	-	-	-	+
N-acetyl-D-Mannosamine	-	-	-	-	-	-
Melobiose	-	-	-	-	-	+

- negative + inhibition

Summary table of the Saccharides tested with each Monoclonal Antibody or Capon Antiserum and their Inhibitory Effects on the Antibodies. TABLE 2