Chemical characterization of *Mycoplasma dispar* capsule and a study

of its immune responses in calves and mice.

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GENERAL INTRODUCTION

Mycoplasma dispar is a pathogen of the respiratory tract of calves that is known to cause chronic colonization of the tracheal mucosa. Although *M. dispar* rarely causes mortality by itself, it is known to predispose the animal to more severe infections with highly pathogenic bacteria such as *Pasteurella hemolytica.* This predisposing role is most likely related to the impairment of tracheobronchial clearance that has been demonstrated during infection with this mycoplasma (Thomas et al., 1987; Howard and Thomas, 1974).

With the aid of ruthenium red staining, Howard and Gourlay (1974) showed that *M. dispar* produces a polysaccharide capsule (capsular polysaccharide, CPS) during natural infections. The production of this CPS can also be induced in vitro, under conditions of co-culture with bovine lung fibroblasts (Almeida et al., 1991). With CPS of other bacteria, it has been shown that the CPS can help the microorganism in evading phagocytosis. They can do so by conferring surface negative charges (Van Oss and Gilman, 1973) or by masking binding receptors on the microorganism for opsonic factors such as the C3b protein of the complement pathway (Horowitz, 1982). Capsules have also been shown to promote adherence of organisms to the surfaces of inanimate objects or living cells by formation of biofilms (Costerton et al., 1987) and by binding irreversibly to negatively charged surfaces by a latch effect provided by the multiple binding sites from repeating units in the polysaccharides (Robb, 1984). Published work has shown the CPS of *M. dispar* to be involved in the suppression of several alveolar macrophage functions such as phagocytosis, tumor necrosis factor and interleukin-1 production (Almeida et al., 1992). Participation of mycoplasma capsule in the attachment process has been suggested by Howard et al (1974). The association of CPS with immunosuppressive activities and attachment of *M. dispar* to host cells implicates the CPS as a virulence factor.

Several attempts have been made to measure antibody responses to *M.*

dispar protein antigens (Howard and Gourlay, 1983; Howard, 1983. Scott et al., 1980; Thomas et al., 1982) in naturally or experimentally infected cattle but almost none or very poor responses were seen. No work has been published on the immune responses to the capsular antigen in infected calves. There is no information available regarding the antigenic nature of purified CPS of *M. dispar,* i.e. whether it acts as a typical thymus independent type 2 antigen similar to other polysaccharides or whether it has some unique properties associated with it.

There is preliminary evidence that the CPS of *M. dispar* is similar in chemical composition to polygalacturonic acid (pGalU) which is found in citrus rinds and other plant tissues (Almeida et al., 1990). If vaccines based on the CPS provide protection against infection and tissue damage by *M. dispar,* then there is potential for use of inexpensive, commercially available pGalU instead of the CPS of *M. dispar* which is expensive to produce in large quantities.

If the CPS of *M. dispar* does elicit thymus-independent responses, then in order to induce immunity in animals, thymus-dependent (TD) forms of the CPS will have to be constructed. Such TD forms will enable the host's immune system to elicit an anamnestic response, produce antibodies with much higher affinity for CPS and also produce IgG antibodies which can be transferred to the new-born through placenta and colostrum. One way to construct such forms of the CPS would be through chemical modifications and conjugation with a protein. However, such approaches to vaccine development require understanding of the chemical structure of CPS.

The main objectives of this research project were 1) to characterize the immune responses towards pGalU and to the purified capsule of *M. dispar* in mice, 2) to characterize the immune responses to capsule and pGalU in calves infected with *M. dispar,* and 3) to chemically characterize the capsule of *M. dispar.*

Thesis organization

This thesis is composed of a general review of the literature followed by two papers being submitted for publication to different journals. A summary of the entire thesis and one appendix are included. The references cited in the General Introduction, Literature Review and General Summary follow the General Summary.

LITERATURE REVIEW

This literature review covers the area of bacterial capsular polysaccharides, including their structure and function. In concordance with the focus of the research, special emphasis has been laid on the immune responses towards polysaccharides.

Bacterial extracellular polysaccharides

Carbohydrates are universally present on the surface of living cells. On eukaryotic cells, many different carbohydrates are attached as glycoproteins and glycolipids; the oligosaccharide moieties are known to act as receptors and they are known to play an important role in cell to cell recognition processes. In prokaryotes, polysaccharide capsules characteristically composed of repeating oligosaccharides are found on the surface of many bacteria.

Bacterial extracellular polysaccharides (EPS) are found in one of two forms. As a capsule (capsular polysaccharide, CPS), the polysaccharide is intimately associated with the cell surface and may be covalently bound but is distinct from and extraneous to the bilayer membrane. In contrast, slime polysaccharides are only loosely associated with the cell surface. Distinction between CPS and slime polysaccharides is operationally defined by the degree of cell association following centrifugation (Whitfield, 1988). Differentiation between the two forms may be difficult, since cells producing large amounts of CPS may release some material at the periphery, giving the appearance of slime production. Stable mutants that are no longer able to attach the polysaccharide in the form of capsules are found. It is not clear whether the mutants lose a transferase type of enzyme which is involved during the final stages of capsule formation or whether an attachment site on the cell surface is lost. Although no chemical differences have been reported between the capsule and slime polysaccharides prepared

from wild type strains and their slime-forming mutants, for *Enterobacter aerogenes* it was shown that the slime polysaccharides had a lower molecular weight than the capsular material (Wilkinson, 1958). Most bacteria show a preference of producing one form over another, although some strains of *Klebsiella* sp. and *Staphylococci* sp. (Wilkinson, 1983) can simultaneously produce identical capsule and slime. Several bacteria including strains of *Rhizobium* sp., *Agrobacterium* sp. and *Alcaligenes facalis* var. *myxogenes* are able to synthesize more than one chemically distinct exopolymer (Sutherland, 1985). Distinction between CPS and other cell surface polysaccharides can also be difficult. The CPS K-antigens of some *Escherichia coli* K serotypes are now known to be lipopolysaccharides.

Among the gram-positive cocci, streptococci and staphylococci have been reported to produce polysaccharide capsules. These include *Streptococcus pyogenes, Streptococcus agalactiae, Streptococcus suis, Streptococcus pneumoniae, Streptococcus bovis, Streptococcus mutans, Streptococcus salivarious, Staphylococcus aureus* and *Staphylococcus epidermidis. Bacillus anthracis*, a gram-positive rod also produces a capsule. *Neisseria meningitidis* and *Neisseria gonorrhoeae* (Hendley et al.,1977) are the gram-negative coccobacilli that have been reported to possess capsules. Examples of encapsulated gram-negative rods are *Escherichia coli, Salmonella typhi, Salmonella paratyphi* C, *Salmonella dublin*, members of *Klebsiella* species, *Vibrio parahaemolyticus, Haemophilus influenzae, Pasteurella multocida, Pasteurella haemolytica* and *Bacteroides fragilis.*

The production of the EPS may be affected by growth conditions. Synthesis of alginate by mucoid strains of *Pseudomonas aeruginosa* is a growth - associated process and the specific rate of its production in continuous culture increases with increased specific growth rate (Mian et al., 1978). Alginate is produced mainly during the exponential phase of growth (Annison et al., 1987) although EPS synthesis commences with the onset of stationary phase.

Production of this EPS also depends on the temperature of growth, more alginate being produced per cell at 12 C than at 25 C and least at 37 C (Evans et al., 1973). Also, addition of 5% glycerol and 0.25 M NaCl to MacConkeys agar stimulates EPS production at all temperatures. Capsule formation by *Bacillus anthracis* is optimal on special media under 5% CO_2 (Orskov et al., 1990). Lee et al., (1993) reported that there was an inverse relationship between capsule (type 8 CPS) expression by *Staphylococcus aureus* and the iron concentration in the culture media. A similar relationship has been reported in the case of *Neisseria meningitidis* (Masson et al., 1985). Lee et al., (1993) also reported that *S. aureus* produces 350 times more cell-associated CPS per miligram of biomass when grown on the surface of Columbia agar than when grown in Columbia broth. Most of the CPS produced by broth-grown cells was secreted in the culture medium.

Mycoplasma extracellular polysaccharides

Mollicutes lack a cell wall containing peptidoglycan and protein matrices as are found in most prokaryotes (Plackett, 1959). Similar to many bacteria, capsules have been described in several mollicute species. However, morphologically they appear to more closely resemble the glycocalyx of eukaryotes than the classical capsules of the more traditional eubacteria (Minion et al., 1993).

Recently, two comprehensive reviews regarding the capsular polysaccharides of mycoplasmas have been published (Minion et al., 1993; Rosenbusch et al., 1992). Several mycoplasma species produce a measurable capsule including *Mycoplasma mycoides* sub sp. *mycoides* (Buttery et al., 1960), *Mycoplasma dispar* (Howard et al., 1974), *Mycoplasma gallisepticum* (Tajima et al., 1979), *Mycoplasma hominis* (Furness et al., 1976), *Mycoplasma hyopneumoniae* (Horn, 1970; Tajima and Yagashaki, 1982), *Mycoplasma maleagridis* (Green et al., 1973), *Mycoplasma pneumoniae* (Wilson et al., 1976), *Mycoplasma pulmonis* (Taylor-Robinson et al., 1981), *Mycoplasma synoviae*

(Ajufo et al., 1978), *Mycoplasma mobile* (Rosengarten et al., 1988), and *Spiroplasma citri* (Cole et al., 1973). Among the ureaplasmas, the human pathogen *Ureaplasma urealyticum* expresses capsule (Robertson et al., 1976), but *U. diversum* has been described to have only a very thin exopolymer (Boatman et al., 1976). Rurangirwa et al. (1987) have reported the production of an extracellular polysaccharide from *Mycoplasma capricolum* with a large molecular weight. Although no morphological data is available, it has been considered as the capsular polysaccharide (Minion et al., 1993) in view of its molecular weight which has been shown to be greater than 200 Kd. Capsules have not been described among acholeplasmas, spiroplasmas, anaeroplasmas or asteroplasmas and therefore may be considered unique to mammalian pathogenic species.

The traditional methodology used for studying bacterial capsules has not been applicable with great success to mollicutes because of their smaller size and the complex nature of background components provided by the media or animal tissue environment in which capsule is being expressed.The majority of the morphological information available has arisen from electron microscopy observations. Mostly, mycoplasma capsules have been visualized with the aid of polycationic compounds such as ruthenium red and polycationic ferritin which complex with osmium tetroxide to stain polyanionic compounds (Luft, 1964). Rosenbusch and Minion (1992) have raised the question whether capsular structures that are either not polyanionic or are not reactive with polycationic compounds exist among mycoplasmas but have not been reported.

Lipoglycans (Smith, 1984) have been found in the members of these mollicute families, but these structures are considered integral to the mycoplasmal membrane (Minion et al., 1993). These lipoglycans can be extracted by treatment of the whole cells or membranes alone with hot 45 % phenol (Smith, 1984). Therefore, capsular material that has been isolated by the use of hot phenol extraction procedures may be contaminated with lipoglycans. The use of milder

treatments such as prolonged exposure to buffered saline at 37° C for the extraction of capsule (Almeida et al., 1992) would not be expected to remove the lipoglycans from the mycoplasmal membrane. Moreover, lipoglycans do not provide significant extracellular electron microscopic image when stained with ruthenium red and are of lower molecular weight than capsules (Rosenbusch et al., 1992).

Howard and Gourlay (1974), demonstrated capsule on the surface of *M. dispar* (grown in glucose - calf serum - broth) by ruthenium red staining. The *M. dispar* capsule extended about 17-24 nm and *M. mycoides* capsule about 30 nm beyond the cytoplasmic membrane. Similar to *M. mycoides* subsp. *mycoides* and *M. meleagridis*, the *M. dispar* capsule possessed no obvious structure. Thus the ultrastructure of the mycoplasma capsule is distinct from those of *Diplococcus pneumoniae*, where the capsule was found to have a fibrous structure and *Klebsiella pneumoniae* , where a spike or net-like appearance was observed (Springer and Roth, 1973). With the aid of ruthenium red staining, mycoplasma capsules could be seen as amorphous layers. Capsules of up to 40 nm in thickness were seen surrounding *M. hyopneumoniae* in infected porcine lung tissue (Tajima et al., 1982).

Little is known concerning the effects of environmental factors on capsule production. In some species, capsule seems to be produced constitutively whereas others synthesize the capsular polysaccharide to a considerable extent only during *in vivo* growth. *M. dispar* produces little capsule in vitro but produces significant amounts *in vivo* or when cocultured with bovine lung fibroblasts (Almeida et al., 1991). Increases in the thickness of the capsule have been shown to occur with cocultured *M. gallisepticum* (Tajima et al., 1979) and *M. hyopneumoniae* (Tajima et al., 1982). The nature of the interaction occurring between eucaryotic cells and the mycoplasmas is still undefined, but in the *M. dispar* model it has been shown that a dialysis membrane with a 14,000 molecular weight cut-off can be interposed between cells and mycoplasmas

without interfering with the induction of capsule synthesis (Almeida et al., 1991).

Structures of bacterial and mycoplasma polysaccharides

Methods for structural elucidation of polysaccharides

The immunogenicity and the immunological specificity of polysaccharide antigens is dictated by their structures. Hence, in order to understand the immunology of polysaccharides and to develop effective prophylactic vaccines against encapsulated bacteria, it is imperative that the structure of the polysaccharide capsule be elucidated.

Many chemical techniques have been utilized to determine the structures of the bacterial and mycoplasma capsules.

Thin-layer chromatography is generally used in conjunction with acid hydrolysis and enzymatic cleavage to determine the monosaccharide composition. Rurangirwa et al (1987) determined the composition of the polysaccharide obtained from F-38 strain of mycoplasma (etiologic agent of contagious caprine pleuropneumonia) by thin layer chromatography of the trifluoroacetic acid hydrolyzed polysaccharide. The acid hydrolyzed polysaccharide consisted of glucose, galactose, mannose, fucose, glucosamine and galactosamine in approximately equal quantities.

Methylation analysis of polysaccharides has been used to provide information regarding the amount and the point of branching in the polysaccharide. The free hydroxyl groups in the polysaccharide are methylated by reaction with methylsulfinyl carbanion and methyl iodide. The methylated polysaccharide is then hydrolysed, and the resulting partially methylated monosaccharides are reduced, acetylated and analyzed by gas chromatography coupled to mass spectrometry. Rodriguez et al (1988) methylated the carboxyl-reduced capsular K4 antigen of E. coli O5:K4:H4 and found that there were 3 partially methylated polyol acetates in the mixture: 2,5-di-O-acetyl-1,3,4,6-tetra-O-

methylmannitol; 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol and 1,3,4,5-tetra-O-acetyl-2,6-di-O-methylglucitol. From these results, the authors inferred the presence of 3,4-linked glucuronic acid, 3-linked N-acetylgalactosamine and terminal fructofuranose in the K4 polysaccharide.

Another technique that has been used with great success in determining the structures of polysaccharides is nuclear magnetic resonance spectroscopy . This technique has many advantages over other chemical degradation techniques. NMR spectroscopy is not destructive of the sample whereas all the other techniques are. Besides, other techniques can only provide partial information regarding the structure of the capsule whereas structures of many of the bacterial polysaccharides have been deduced almost entirely by NMR spectroscopy (Bhattacharjee et al., 1975; Crisel et al., 1975). The first NMR spectroscopic technique to be exploited was ^{13}C NMR which yielded valuable information on the structure and conformation of polysaccharides (Jennings et al., 1977; Egan, W., 1980; Bundle et al., 1974). A spectra obtained on ^{13}C NMR spectroscopy of bacterial polysaccharides contains signals associated with all the individual carbon atoms of their basic skeleton. Despite the large number of carbons in these molecules, the pattern of these signals is simplified by the coincidence of the carbon signals of their individual identical repeating units. Bhattacharjee et al (1975) performed ^{13}C NMR on the group B meningococcal polysaccharide. Despite the large molecular size of the polysaccharide, the NMR spectrum was found to be a eleven-resonance spectrum, containing one signal for each carbon in its α-(2-->8)-linked sialic acid repeating unit. Thus the simple pattern of the signals provided good evidence for the group B meningococcal polysaccharide to be composed of the above repeating unit. In addition, further structural information was obtained by comparing the chemical shifts of the carbon atoms of methyl α- and β- ketosides of sialic acid with those of the sialic acid residues in the polysaccharide. Large chemical shift differences at C-8 and

smaller ones at C-7 and C-9 confirmed that the sialic acid residues were linked at O-8, and similarities in the chemical shifts of C-1, C-4 and C-6 of the methyl α-ketoside with those of the sialic acid residues in the polysaccharide enabled the α-configuration to be assigned to these residues. Also, chemical shifts have also been demonstrated to be conformationally sensitive (Bundle et al., 1974). Thus, overall 13C NMR spectroscopy has proved to be a powerful technique in the structural determination of bacterial polysaccharides.

As in the case of 13C-NMR spectra, the 1H-NMR spectra of polysaccharides are considerably simplified due the presence of a single repeating unit. However due to the presence of a large number of protons, the one-dimensional 1H-NMR spectra are generally more poorly resolved as compared to 13C-NMR spectra. The chemical shifts of the proton signals can be used to make proton assignments , and changes in chemical shifts following specific chemical modifications of polysaccharides can be used to provide valuable conformational information. The added advantage of 1H-NMR spectroscopy is due to the phenomenon of proton-proton coupling, from which the relative orientation of vicinal protons can be established. However, because of the complexity of most one-dimensional 1H-NMR spectra, this technique is usually used for assigning anomeric proton signals which resonate in the characteristic low field part of the spectrum.

The ability to make assignments to the peaks has been greatly facilitated with the advent of two-dimensional techniques. These techniques have been used for the structural elucidation of the groups I (Michon et al., 1985a) and K (Michon et al., 1985b) meningococcal polysaccharides. By performing proton homonuclear shift-correlated 2-D NMR (1H-1H COSY) and heteronuclear shift-correlated 2-D NMR (13C-13C COSY) experiments, the investigators were able to make unambiguous assignments to all the protons and carbons of the repeating units.

Structures of capsular polysaccharides

The capsular polysaccharides produced by microorganisms are cell-surface polymers consisting of repeating oligosaccharide units. They may be linear homopolymers of a single carbohydrate moiety, e.g. meningococcal groups A,B and C polysaccharides; linear heteropolymers composed of two or more monosaccharides, such as pneumococcal types 3 and 19F polysaccharides; or they may be multi-chained polymers composed of two or more monosaccharides and additional moieties, e.g. O-acetyl, pyruvic acid, glycerol, ribitol and phosphodiester bonds such as pneumococcal types 1, 4, 18C, 6 and 19A polysaccharides.

Based on the type of the capsular polysaccharide present, *N. meningitidis* has been classified serologically into groups A, B, C, 29e, W135, X, Y and Z. Group A PS is a homopolymer of 2-acetamido -2-deoxy D-mannopyranosyl phosphate (Bundle et al, 1974) and group X is a homopolymer of 2-acetamido -2-deoxy D glucopyranosyl phosphate (Bhattacharjee et al., 1975). Group B and C PSs are homopolymers of sialic acid. The groups Y and W135 contain D-glycosyl and D-galactosyl residues respectively in addition to sialic acid. The group Z contains glycerol-3-phosphate in addition to 2-acetamido -2-deoxy -D-galactopyranose residues.

According to their type specific capsular polysaccharides, *H. influenzae* can be classified serologically into six types (a through f). Types a, b, c and f contain phosphodiester linkages. Types d and e contain N-acetyl mannuronic acid. All of *H. influenzae* polysaccharides are high molecular weight negatively charged surface polymers. Except for type e, all types have a relatively simple structure, being a D-ribofuranosyl -D- ribitol phosphate polymer (Crisel et al., 1975).

Capsular antigens of *E. coli* are acidic polysaccharides with different chemical compositions which have been grouped into two kinds depending upon the molecular weight and the acidic components present. The type I capsular polysaccharides have molecular weights greater than 100 Kd and the acidic

components are glucuronic acid, galacturonic acid and pyruvate whereas the type II polysaccharides are less than 50 Kd and contain glucuronic acid, N-acetylneuraminic acid, phosphate and 2-Keto-3-deoxymanno-octonic acid (KDO) as their acidic components. The structures of the capsular K polysaccharides have been reviewed by Jann and Jann (1990).

The structures of the capsular antigenic polysaccharide from most of the eighty types of the gram-negative bacterium *Klebsiella* have been elucidated and a complete account of them has been reported (Isaac, 1985). *Klebsiella* K5 capsular polysaccharide is a linear trisaccharide repeat including two charged groups, namely a glucuronic acid residue and a 4,6-ketal pyruvate attached to the mannose residue. An O-acetate is attached at the 2-position of the glucopyranose ring. *Klebsiella* K8 is a tetrasaccharide sequence consisting of three neutral sugar residues in the backbone and a charged glucuronic acid residue in the side chain. *Klebsiella* K9 is a pentasaccharide repeat consisting of four neutral sugar residues (three L-rhamnose residues and one D-galactose residue) in the backbone and one charged glucuronic acid residue as the side appendage. The chemical repeating sequence of the *Klebsiella* serotype K18 is the most complex of *Klebsiella* polysaccharides. It is a polyhexasaccharide consisting of a trisaccharide backbone repeat with a trisaccharide side chain attached to the α-D-glucose residue of the backbone. The only charged group in the repeat is the carboxyl of the β-D-glucuronic acid moiety which is the middle residue of the side chain. Another interesting polysaccharide is that of serotype K25 which consists of a tetrasaccharide repeat with a polydisaccharide backbone and a disaccharide chain attached. The backbone is similar to the animal connective tissue polydisaccharide, hyaluronic acid, the chondroitin sulfates and dermatan sulfate. The charged group in the repeat is the carboxyl of the glucuronic acid residue which is attached directly to the backbone.

Among *S. pneumoniae* strains, 83 type specific polysaccharides are

produced which have been designated 1-83 in the U.S. system. The Danish system has combined closely related types into groups. Most pneumococcal polysaccharides are negatively charged and possess acidic components, including D-glucuronic acid (e.g. types 2, 3, 5, 8, 9A, and 9V), D-galacturonic acid (e.g. type 1) and phosphate in phosphodiester bonds (e.g. types 6A, 6B, 10A, 11A, 15B, 19F, 19A, 20 and 23F). Type 4 polysaccharide contains pyruvate as its acidic component, while type 12F contains 2-acetamido-2-deoxy-D-mannuronic acid as the acidic component. The structures of pneumococcal polysaccharides have been reviewed (Jennings 1990). One property of the pneumococcal polysaccharides that enabled limitation of their number in the vaccine was their extensive serologic cross-reactivity demonstrated in animal experiments. The origin of this cross-reactivity is the extensive structural homology found in the pneumococcal polysaccharides, which is exemplified in the Danish serotyping system.

Group B *streptococci* produce four type-specific polysaccharides: Ia, Ib, II, III and IV. The structures of all but the last have been elucidated (Jennings et al., 1983a; Jennings et al., 1983b; Wessels et al., 1987). All the polysaccharides contain D-galactose, D-glucose, 2-acetamido-2-deoxy-D-glucose and sialic acid. This 3-O-(N-acetyl-α-neuraminyl)-β-D-galactopyranosyl group of these polysaccharides is the end-group oligosaccharide in the human M and N blood group substances and thus the organism can effectively evade the human immune system (Jennings, 1990).

The structure of the capsular polysaccharide of *M. mycoides* subsp. *mycoides* and *Mycoplasma* sp. bovine arthritis strain have been elucidated. The polysaccharide is a galactan of unusual structure consisting of galacto-furanosyl units in 1->6 β linkage (Plackett and Buttery, 1964). The predominant structural unit of the glucan from the bovine arthritis strain is β-D-glucopyranosyl-(1->2)-D-glucopyranose (Plackett et al., 1963).

Function of the capsules

Capsules are important determinants of the behaviour of bacteria within the animal host. Comprising 99 % of water, these highly hydrated polysaccharide capsules may serve many functions most of which are protective in nature. Capsules may enhance survival and facilitate spread of bacteria from one host to another by preventing dessication. They may significantly affect the access of molecules and ions to the bacterial cell envelope and the cytoplasmic membrane (Dudman,1977). Cells buried within a polymer matrix, may, for instance, be inaccessible to antibacterial agents such as antibiotics (Costerton et al., 1987).

Capsules may promote adherence of bacteria to the surfaces of inanimate objects or living cells by formation of biofilms (Costerton et al., 1987) and by binding irreversibly to negatively charged surfaces by a latch effect provided by the multiple binding sites from repeating units in the polysaccharides (Robb, 1984). Association between attachment and virulence of mycoplasma species was reported by several authors (Gabridge, 1983; Bredt et al., 1981) and participation of the mycoplasma capsule in the attachment process was suggested (Green and Hanson, 1973; Wilson and Collier, 1976; Howard et al., 1974). Electron microscopy pictures of *M. dispar* attached to RBC have shown the presence of ruthenium red stainable capsular material and fine threads of extracellular material bridging gaps between membranes. The author suggested that this was evidence of participation of *M. dispar* capsule in the attachment process (Howard et al., 1974). Similar observations and suggestions were made by Tajima and Yagihashi (1982) in their study of the interaction of *Mycoplasma hyopneumoniae* with porcine respiratory epithelium using transmission electron microscopy.

Among certain gram-positive and gram-negative bacteria, capsules have evolved distinctive structural and functional characteristics which are of cardinal importance in the pathogenesis of infection of animals, plants and insects.

Capsulated bacteria are responsible for causing some of the most serious invasive infections to which man is susceptible, including septicemia, meningitis, pneumonia, osteomyelitis, septic arthritis and pyelonephritis. The morbidity and mortality caused by these infections is substantial and their capsules have been implicated as the major virulence factors (Robbins, 1978). The type of EPS , the amount synthesized and the rate of synthesis may all have a bearing on the pathogenicity of an organism.

Capsules seem to be particularly important in bacteria whose strategy for survival in the host depends on the evasion of phagocytosis (Dudman, 1977; Horowitz and Silverstein, 1980; Jann and Jann, 1983). Most capsular polysaccharides are hydrophilic and confer a negative charge on the bacterial cell, characteristics which are intrinsically antiphagocytic in their effect. The experimental observations of Ponder (1928), Van Oss and Gillman (1973), and Absolom (1988) were the basis of the assumption that the hydrophilic properties of polysaccharide capsules act by reducing the surface tension at the interface between the phagocytic cell and the bacterium. This impaired the ability with which phagocytic ingestion occurs. In biological fluids containing IgG, most non-encapsulated microorganisms, because of their hydrophobic surface (increased aqueous surface tension) non-specifically adsorb IgG. It was found that an IgG coat and the subsequent complement activation with deposition of C_{1423} enhances the surface hydrophobicity which increased the phagocytic engulfment of microorganisms by the PMNs. Encapsulated microorganisms, which were hydrophilic did not absorb IgG at their periphery to any significant extent and failed to induce efficient phagocytic ingestion. Because of the surface charge, the contact between the capsulated bacteria and the phagocytic cell is compromised since the negative charge on each cell results in mutual repulsion. Thus, due to the physical properties of CPS including hydrophilicity and charge, the phagocytic ingestion of capsulated organisms is inefficient in the absence of factors which facilitate contact between the bacterium and the phagocytic cell and which modify the

hydrophilic bacterial surface.

Microorganisms have been found in host systems forming microcolonies immersed in a glycocalyx which creates a protective niche where individual microorganisms survive host defense mechanisms. Isenberg (1988) reported that the microorganisms were immersed in a protective halo made up of joined individual exopolysaccharides which were resistant to degradation by mammalian enzymes. Consequently, phagocytes were unable to break the exopolysaccharide barrier to engulf these microcolonies.

Many host factors can act as opsonins which modify the surface of capsulated bacteria, but C3b - the cleavage product of the third complement component- is of central importance, especially against invasive infections caused by capsulate bacteria (Winkelstein, 1981). C3b can be generated by two independent mechanisms, the alternative and classical pathways. The formation of C3b through the alternative pathway is of particular importance in the non-immune host; in the early phase of invasive infection with capsulated bacteria, specific antibodies are absent and therefore antibody-independent activation of C3 and the deposition of C3b on the bacterial surface is a major source of opsonic activity. The C3b deposited on the bacterial surface can act as a ligand of specific receptors on polymorphonuclear leukocytes or macrophages. Activation of C3 triggers the complement cascade leading to the formation of the MAC (membrane attack complex), whose fixation on the surface of the bacteria can cause bacteriolysis. Factor H is a complement regulatory protein which competes for surface bound C3b with factor B (Fearon 1978) and facilitates the degradation of C3b by factor I. This disassembly of the C3 convertase terminates the C3b amplification loop and limits the deposition of this important opsonin on the bacterial surface. Bacterial capsules have evolved to take advantage of this competition between factor B, which promotes amplification and factor H, which terminates it. This has been best studied in type III, group B streptococci (Edwards et al., 1982) and in the K1-encapsulated E. coli (Stevens et al., 1978). Both

capsules contain sialic acid , which increases the affinity of factor H for cell-bound C3b. A similar strategy of limiting complement deposition has been described for capsules that do not contain sialic acid. The capsules of type 7 and type 12 pneumococci have a decreased binding affinity for factor B, which results in a relative increase in the binding of factor H (Joiner et al., 1984). The streptococcal M protein, a surface fibrillar molecule has a high affinity for factor H and thereby evades killing mediated by the alternative pathway (Horstmann et al., 1988).

Masking of microorganism binding receptors for opsonic factors by the capsular layer has also been suggested. Capsule may be permeable to opsonic factors that recognize sites beneath the capsular surface, but this structure may present a physical barrier that prevents the opsonic ligand from contacting the phagocytic cell (Horowitz and Silverstein, 1980; Horowitz, 1982). Verbrugh et al. (1982) showed that IgG from nonimmune serum and C3 were deposited beneath the capsule of *Staphylococcus aureus* and evidently were blocked from interacting with the receptors on the phagocytic cells: anticapsular antibody promoted C3 deposition throughout the capsule, including the bacterial surface, and resulted in efficient opsonization and phagocytosis.

The onset of the immune response can drastically increase the efficacy of the host defense.There are, however, situations in which the immune state of the host vis-a-vis an infecting microorganism is virtually never reached and susceptibility to infection is maintained in late stages of an infection. Well known examples are extraintestinal infections with *E. coli* exhibiting the K1- or K5-specific capsular polysaccharides. This is because the structure of K1 polysaccharide is identical with the terminal carbohydrate region (Finne, 1982; Hoffmann et al., 1982) of the embryonic form of the neural cell adhesion molecule. Similarly, the structure of the K5 polysaccharide is identical with the first polymeric intermediate in the biosynthesis of heparin (Navia et al., 1983). A similar example of structural mimicry has been reported with *Mycoplasma mycoides* subsp. *mycoides* . It has been suggested that the mycoplasma galactan possesses

serological similarity to pneumogalactan, a product of the normal lung epithelial cells (Gourlay and Shifrine, 1966; Shifrine and Gourlay, 1965). Structural analysis of the capsular K4 antigen (Rodriguez et al., 1988) revealed that this polysaccharide had the structure of chondroitin, substituted by fructose. This fructose substituent, which was found to be the immunodominant sugar of the K4 polysaccharide, occurs in such a labile linkage that it is removed at pH 4.0 at 37 C. In buffered cultures of *E. coli* O5:K4:H4, the polysaccharide capsule was found to lose its fructose constituent with the conversion to nonimmunogenic chondroitin. Thus, growth of this *E. coli* strain in body compartments of low pH may convert encapsulated bacteria from a form in which they induce and react with specific anticapsular antibodies to a form in which they can no longer do so.

Since efficient phagocytosis requires the presence of specific anticapsular antibody, opsonization of encapsulted bacteria can be hampered by poor immunogenicty of the CPS.

Types of immunological responses

Specific immune responses are classified into two types, based on the components of the immune system that mediate the response: Humoral immunity and cell-mediated immunity.

Exposure of the immune system to foreign antigens sets into motion the series of events that lead to lymphocyte activation and the generation of humoral and cell-mediated immunity. Different antigens and conditions of immunization lead to responses that vary both quantitatively and qualitatively. The magnitude of an immune response to an antigen is determined by a balance between lymphocyte activation and tolerance induced by that antigen. In fact, the same antigen can be administered in ways that preferentially stimulate lymphocyte growth and differentiation or inactivate lymphocytes and induce functional unresponsiveness. The nature of an immune response to an antigen is

determined by the specificities and functional classes of lymphocytes that are activated by that antigen. For instance, different antigens preferentially stimulate the production of antibodies of various heavy chain isotypes or generate CTLs or other effectors of cell-mediated immunity. Such variability is important because it enables the immune system to protect an individual from the many distinct types of microbes present in the environment.

Antigens being the obligatory first signal for lymphocyte activation, the nature of the antigen has significant influence on the type and magnitude of the immune response that develops.

Classification of antigens

One of the important findings leading to the discovery of T and B cells was the realization that most antigens required both cell types for induction of an antibody response. It was soon found that this was not the case with all antigens. Thus, LPS (Moller et al., 1971) and polyvinylpyrrolidone (Andersson et al., 1971) could induce an immune response without the help of T cells. On this basis, antigens were classified into two major classes: thymus dependent (TD) and thymus independent (TI).

Thymus dependent (TD) antigens consist of soluble proteins such as hemocyanin, ovalbumin, bovine gamma globulin, haptenated derivatives or bacterial proteins such as tetanus and diphtheria toxoids or whole cells, viruses or parasites. They require the presence of mature T lymphocytes , which have been procesed by the thymus gland, as well as antigen presenting cells.

Antigens were classified as thymus independent (TI) because they were able to elicit responses in congenitally athymic nude mice or in adult mice who had been thymectomized in the newborn period. Thus these antigens do not require the presence of the thymus or mature T lymphocytes to stimulate an antibody response. However, their behaviour as TI antigens does not mean that they cannot be influenced by T cells, either directly or by cytokines produced by

the T cells (Mongini et al., 1981). A common characteristic of these molecules was that they consisted of repeated antigenic determinants. This property was considered to explain their T-cell independency: the repeating antigenic determinants should result in an efficient crosslinking of the immunoglobulin membrane receptors on the B cells and this should give activation signals to the B cells. This is the view held by majority of the immunologists today. However, two other views on the nature of TI antigens have been proposed. One is simply to deny their existence: TI antigens are only less T-cell dependent than other antigens (Cohn et al., 1975). The other view is that all TI antigens are polyclonal B cell activators and this triggers B cells that have bound the antigen by their specific immunoglobulin receptors (Coutinho et al., 1974).

There are two types of TI antigens (Mosier et al., 1982). This classification was based on the observations that neonatal mice (Mosier and Zaldivar et al., 1977) and mice linked with an X-linked genetic defect such as the CBA/N mouse strain (Amsbaugh et al., 1972) failed to respond to soluble capsular polysaccharides but did respond to other TI antigens. When the properties of the group of TI antigens which could stimulate CBA/N spleen cells (TNP-BA, TNP-LPS, high epitope TNP-beads) were further analyzed, it became clear that these antigens were dissimilar in many respects. Whereas in vitro responsiveness of cells to TNP-BA and TNP-LPS was acquired at birth (Mosier et al., 1977), responsiveness to high epitope bead was acquired at 2 to 3 weeks of age (Mond et al., 1979) and in this regard was similar to acquisition of responsiveness to TNP-Ficoll (Mosier et al., 1977). Furthermore, TNP-BA (Mond, 1982) and TNP-LPS (Wong and Herscowitz, 1979) were found to be macrophage independent antigens while TNP-polyacrylamide beads (Mond, 1982) were found to be macrophage dependent. Although experiments in vitro utilizing spleen cells from nu/nu mice indicated that TNP-Ficoll behaved as a TI antigen, rigorous depletion of T cells from spleen cells of euthymic mice resulted in the abrogation of the response to the antigen and this response was reconstitutable with purified

populations of T lymphocytes (Mond, 1982). Thus, TI-1 antigens require least amout of T cell help and can elicit responses in CBA/N mice as well as in neonatal mice. The TI-2 antigens, on the other hand, are unable to stimulate B lymphocytes without ancillary help. Despite this, these antigens cannot be regarded as TD antigens since they do stimulate responses in nude mice.

Type 1 TI antigens are bacterial products, tend to have lipids attached, function as polyclonal activators in the mouse, activate the alternate complement pathway and stimulate responses in the neonates and the *xid* mice; type 2 antigens tend to be high-molecular-weight polymers with repeating determinants and cannot stimulate responses in the neonates or the xid mice. Examples of type 1 TI antigens would include lipopolysaccharide and haptenated derivatives, *Brucella abortus* and haptenated derivatives, *Nocardia* water-soluble mitogen and haptenated derivatives, polymerized flagellin from *Salmonella* bacteria, *N. meningitidis* heat-killed bacteria and outer membrane proteins. Examples of type 2 TI antigens would include ficoll and haptenated derivatives, levan, dextran and haptenated derivatives, Pneumococcal polysaccharides, *H. influenzae* polysaccharides, *N. meningitidis* polysaccharides and polynucleotides such as poly I- poly C (Howard et al., 1971; Feldman et al., 1971; Miranda, 1972)

In practice, the assignment of a given antigen to the TI class always is the result of a negative experiment (the best available method of T-lymphocyte depletion fails significantly to reduce the immune response to the antigen), so it is always formally possible that a TI antigen will later be reclassified as TD but not vice versa.

Immunological tolerance

Tolerance (or immunological unresponsiveness) can be defined as a specific depression of the immune response induced by previous exposure to the antigen. This definition would implicitly exclude genetically controlled inability to

respond or an inherent non-immunogenicity of a particular material. Thus, tolerance represents an induced depression in the response of an animal that, had it not been subjected to the tolerance inducing procedure, would be competent to mount an immune response to the antigen being studied. It follows from this definition that any formal experimental demonstration of tolerance requires two exposures to the antigen: an initial tolerance-inducing exposure and a subsequent "challenge" with the antigen presented under appropriate immunizing conditions. Tolerance would be assayed as a depression of the immune responses of the subjects that received both the tolerance-inducing and challenge exposure to the antigen as compared with the responses of subjects that received only the challenge. Tolerance can be either complete, that is no detectable antibody synthesis, or partial, that is, a quantitatively reduced immune response.

TI antigens have been known to possess the ability to tolerize B cells. The characteristics of TI antigens that make them potential tolerogens are the polymeric structures with repeating determinants, their high molecular weight and their slow metabolism in the host's body.

The first step in immunity or tolerance at the cellular level is the binding of antigen to immunoglobulin receptors on the cell. In their studies with hapten-coupled TI antigens, Wilson & Feldmann (1973) and Klaus (1975) established that these antigens, unlike TD antigens, rapidly establish high-avidity binding to B cells, because of multipoint attachment of their repeating antigenic determinants. The epitope density is a major factor in causing tolerance (Feldmann,1972; Desaymard & Feldmann, 1975). They found that lightly substituted antigens (with 0.6 - 2 epitopes /50,000 molecular weight) were immunogenic but not tolerogenic; molecules with 2 -3 epitopes per 50,000 molecular weight were both immunogenic and tolerogenic; while those with even higher epitope density are obligate tolerogens. However, even lightly substituted antigen (0.6 epitopes/50,000 molecular weight) was as effective as were preparations with a

four-fold higher epitope density in tolerizing primed B cells (Klaus & Humphrey, 1975). Based on these observation together with the observations that low doses of the antigen were required to tolerize these cells (Klaus & Humphrey, 1975), the authors suggested that primed B cells have receptors with higher avidity for antigen than the virgin cells and thus the population of memory B cells from primed adult mice can be tolerized more easily than can the population of potentially responsive mature B cells in unprimed mice.

TI antigens are generally high molecular weight and have long chains, as distinct from the globular nature of TD antigens.The TI antigens must be above a critical size to function as tolerogens. For example, the tolerogenicity of dextran B512 disappears when its molecular weight is reduced from 70,000 to 20,000 (Howard, Vicari and Courtenay, 1975) and that of B2-6-linked polyfructose when reduced from 6000 to 3000 . Moreno, Courtenay and Howard (1976) found that the fructose polymers were able to retain their activity even when they are cut down to much smaller pieces as compared to their glucose counterparts. They suggested that this could reflect stronger binding at individual receptor sites.

TI antigens characteristically persist in the body for a long time, because of the absence of the appropriate catabolic enzymes. The tolerogenicity of D-amino acid polymers, in contrast with the corresponding L-polypeptides, has been attributed to their nonmetabolizable nature. Persistence is important in induction and maintenance of tolerance because it allows high concentrations to be sustained in the environment of the cell.

The mechanism of B cell tolerance induction could be due to immobilization of receptors under conditions where the B cell is unable to clear such an antigen lattice from the membrane (by shedding or endocytosis), as it would normally do during the events leading up to triggering (Klaus, 1976). Alternatively, Sidman & Unanue (1975) suggested that unresponsiveness may result from an intracellular inhibition of receptor resynthesis, following receptor modulation by persisting antigen.

Immune response to bacterial polysaccharides

Ontogeny

One of the major problems in developing effective vaccines for the prevention of diseases caused by encapsulated bacteria such as *S. pneumoniae*, *H. influenzae* and *N. meningitidis* has been the low immunogenicity of their CPS, especially in children under 2 years of age who are at greatest risk for infection with these bacteria. Normal infants under 2 years have a low and inconsistent antibody response to the polyvalent pneumococcal polysaccharide vaccine compared to older children and adults (Borgono et al., 1978; Cowan et al., 1980; Sell et al., 1981). Cowan et al (1980) showed that antibody titers achieved in infants immunized before 23 months of age were not statistically different from infants receiving saline control. Sell et al (1981) demonstrated that the polyvalent pneumococcal vaccine given at 6 months of age stimulated a low level of antibody against *S. pneumoniae* type 3 and the same vaccine given at 12 months of age stimulated low levels of antibody against type 3, 7, 18 and 23. The absolute level of antibody achieved, however was far below that expected in normal adults. Makela et al. (1977) and Anderson et al. (1977) have demonstrated that the antibody response of the polyribose phosphate CPS of *H. influenzae* type b is strikingly age dependent, with adequate levels of postimmunization antibody being achieved only with vaccination after the age of 18-24 months. Gold et al. (1978) and Wilkins et al. (1979) found the similar age-dependent acquisition of antibody responsiveness with meningococcal group A and group C polysaccharides.

This hyporesponsiveness in infants has been shown to be related to the ontogeny of the response to TI-2 antigens. While responses to TD antigens are present at birth, the responses to TI antigens are not. Responses to TI-1 antigens could be seen very early whereas responses to TI-2 antigens could be seen only

3-18 months after birth in humans and 3-6 weeks in mice. Another difference observed between TI-1 and TI-2 antigens was that the adult CBA/N mice and the normal neonatal mice could respond to TI-1 but not to TI-2 antigens. This unresponsiveness of xid mice to TI-2 antigens could be corrected by the transfer of B cells from normal, genetically compatible strains. In addition, reconstruction of thymectomized X-irradiated xid mice with stem cells from normal donors allows the recipients to respond to TI-2 antigens. Both these results (Scher et al., 1975) indicated that the mutant *xid* gene led to B-cell unresponsiveness because of a defect within the B cells or their precursors.Thus the dichotomy observed between TI-1 and TI-2 antigens reflects the differing sensitivities of B-cell subsets at various stages of differentiation, the TI-1-sensitive B cell being an early appearing subset and the TI-2-sensitive subset being a more mature B cell. It was found that the *xid* mice lacked a subset of B lymphocytes characterized by the Lyb-3 (Huber et al., 1977) and Lyb-5 cell surface markers (Ahmed et al., 1977). These cells (designated Lyb-5+ B cells) are present at low frequencies in 2-week-old normal mice and do not reach adult levels until 3-4 weeks of age. In both neonatal mice and mice with xid defect, the failure to respond to polysaccharide antigens correlates with an absence or diminished number of this mature subset of B cells (Mosier et al., 1977). Boswell et al (1980) demonstrated the requirement for Lyb-5+ B cells in the in vitro antibody response to the type 2 TI antigen, trinitrophenylated-Ficoll (TNP-Ficoll).

The utilization of cyclosporin A reinforced the notion that the B lymphocytes can be subdivided into two distinct subsets. Kunkl et al (1980) reported that the administration of cyclosporin A to mice along with antigens blocks the response to TI-2 antigens, leaving the capacity to respond to type 1 TI antigens intact.

Memory and affinity

A significant feature that accompanies the response to a TD antigen is the development of memory cells. Memory B cells (Klinman et al., 1990) are primed

and respond rapidly to a second dose of antigen, resulting in a secondary antibody response that occurs sooner than the primary, is shifted to a higher proportion of IgG than in the primary, and overall is of higher magnitude, usually 10-fold (Stein et al., 1990). In the case of protein antigens or haptens coupled to a protein carrier, the secondary antibodies are of higher affinity than the primary antibodies (Berek et al., 1988; Stein et al., 1980), which is thought to result from antigen selection of antibodies that have undergone somatic hypermutation (Manser et al., 1990). In contrast, a second dose of a TI antigen stimulates a response that is not increased compared to the primary response or is increased only by a small amount , two- to fourfold at most (Stein et al., 1990). In general, the affinities of antibodies to polysaccharides are two orders of magnitude lower than affinities of antibodies to proteins or haptens and there is little or no evidence of affinity maturation . The question of whether antibodies to polysaccharides stimulated by a TD form of the polysaccharide undergo affinity maturation has not been satisfactorily answered.

Class, subclass and combining site

For essentially all humoral immune responses, both IgM and IgG antibodies are produced and represent the major classes. The secondary response to TD antigens is accompanied by an increase in the ratio of IgG to IgM, whereas for TI antigens usually both isotypes are produced in relatively low amounts and in a one-to-one ratio that changes very little with a secondary immunization (Stein et al., 1982; Stein et al., 1990)

In both mice and humans, the predominant subclass in response to a TD antigen is IgG_1; however, IgG_2 is also produced in reasonable amounts. In mice, relatively little IgG_3 and in humans relatively little IgG_3 and IgG_4 are produced in response to TD antigens. In contrast, responses to TI antigens show significant but varying degrees of restriction to certain subclasses. In the mouse, IgG_3, normally expressed at low levels in the serum, is overexpressed among antibodies to

polysaccharide (Stein, 1992). In humans the subclass restriction is not as marked as in mice, but there is a restriction to IgG_2 that is more evident in sera from immunized adults (Shackelford et al., 1988; Shackelford et al., 1987; Rautonen et al., 1986).

Mice with xid-determined xid defect have normal levels of serum immunoglobulins except that IgM levels are moderately depressed (Perlmutter et al., 1979). This, along with the observation that antibodies produced in response to TI-2 antigens are primarily of IgM and IgG_3 classes, has led to the suggestion that IgG_3 production is largely a property of antibody forming cells which develop from Lyb5+ B cells (Kung et al., 1983). Since, TI-1 antigens yield IgG responses in which IgG_1 or IgG_2 predominate, Kung et al (1983) suggested the possibility that when Lyb5+ B cells are activated by TI-2 antigens, a form of Ig switching leading to major expression of IgG_3 predominates and that Lyb5- B cells do not use this switching pathway. Consequently, IgG_3 is a minor fraction of the IgG that is secreted by the Lyb5- lineage.

Responses to protein antigens are polyclonal, probably reflecting the many unique epitopes on these molecules. Polysaccharide antigens, in contrast, are large molecules with repeating determinants and relatively few epitopes. Antibodies to polysaccharides have been found to be oligoclonal rather than polyclonal (Hansburg et al., 1979). Studies of TD forms of polysaccharide have shown that the response is oligoclonal, resembling the TI response; however some diversity is observed (Stein et al., 1982; Insel et al., 1986).

Natural Immunity to polysaccharides

It has been observed that most adult animal sera contain antibodies to polysaccharide antigens of various pathogenic bacteria. Antibodies to pathogenic organisms, e.g. meningococcal group A, B, C, *H. influenzae* type b and pneumococcal type 3 were detected in the animals as they grew or in human

adults without possible contact with these organisms (Robbins et al., 1975; Sutliff and Davies, 1937). Goldschneider et al (1973) found that there was an age-related increase in natural antibodies to the group A meningococcal polysaccharide in children even though the group A organisms were rarely isolated from them. Sell et al (1981), during their study of responses to polyvalent pneumococcal vaccine in infants, found that natural acquisition of type specific antibody occurred in the unvaccinated controls such that by 24 months of age, mean antibody titers of the vaccinated and the unvaccinated groups were not different. On the basis of serological studies, the possible antigenic sources for these serum protective antibodies were identified to be derived from the cross-reactive antigens (capsular polysaccharides) among intestinal and pharyngeal bacteria (Schneerson and Robbins, 1975; Robbins, 1978). This was later confirmed by the recognition of structural similarities between the capsular polysaccharides of various organisms. Bax et al (1988) showed that the capsular polysaccharide of *N. meningitidis* group A cross-reacts with the capsules of *E. coli* K93, E. coli K51 and *Bacillus pumilis*, bacteria that are frequently found in human flora. Similarly, the CPS of *N. meningitidis* group B cross-reacts with *E. coli* K1; CPS of *N. meningitidis* group C with *E. coli* K92 and the CPS of *H. influenzae* type b with the CPS of *E. coli* K100 (Egan et al., 1980). The one exception to the rule that structural similarities form the basis of cross-reacting determinants is the polysaccharide of E. coli K93 which although being highly cross-reactive with the group A meningococcal polysaccharide, does not share with it one single common glycose residue or linkage (Bax et al., 1988). The probable explanation is that the common determinant can be recognized by comparing the two dimension structures of these two polysaccharides (Jennings, 1990). This phenomenon of serological cross-reactions being involved in the human immune mechanism to pathogenic bacteria has been clearly demonstrated by Schneerson and Robbinson (1975). On deliberately feeding non-pathogenic *E. coli* possessing the K100 capsule to human-adult volunteers, they found that intestinal colonization

readily occurred and antibodies specific for the *H. influenzae* type b polysaccharide were induced.

<u>T cell regulation of the magnitude of Ab response</u>

While TI antigens do not require helper T cells for induction of an antibody response, the magnitude of the response may be influenced by suppressor T cells and amplifier B cells. Baker et al (1970) reached this conclusion when they observed that the treatment of thymus bearing mice with antilymphocyte serum (ALS) enhanced the level of response to type 3 pneumococcal polysaccharide.This enhancement could be reversed by transfer of lymphocytes to ALS-treated mice indicating the T cell nature of the suppression. However, the ALS-induced enhancement is not demonstrable in athymic nude mice suggesting that at least a subset of T cells are required for the expression of ALS-induced enhancement. Based on these findings the authors proposed that the antibody response to type 3 pneumococcal polysaccharides were controlled by the activities of 2 kinds of regulatory T cells called suppressor T cells (Ts) and amplifier T cells (Ta). Ts limit the extent to which antibody forming T cells proliferate in response to the antigen, whereas Ta drive B cells to multiply further after antigenic stimulation.

Markham et al (1977), Taylor and Amsbaugh et al (1983), Taylor and Stashak et al (1983) and Taylor et al (1984) deleted Ts or Ta activity from donor cell suspensions by treatment with appropriate anti-CD8 or anti-CD4 monoclonal antibody and complement (since Ts are CD8+CD4- and Ta are CD8-CD4+). They found that on transferring cell suspensions containing both Ts and Ta activity to athymic mice immunized with type 3 pneumococcal polysaccharide, the resulting anti-polysaccharide response is not changed. On the other hand, if Ta activity was eliminated before cell transfer, significant suppression of the response occurred whereas elimination of Ts activity resulted in increased response. Thus, Ts and Ta act in a competitive manner on B cells to control the magnitude of the antibody

response elicited after immunization.

Taylor and Stashak et al (1983) showed that the B cells from mice immunized with type 3 pneumococcal polysaccharide activated antigen-specific Ts cells. Later, Elkins et al (1987) showed that the antigen primed B cells must express cell-surface IgM, but not IgD Ia antigen to be able to activate Ts cells. Elkins et al showed that antigen-primed B cells that were γ-irradiated retained the ability to activate Ts whereas UV treated cells failed to do so. Both γ-irradiated and UV treated antigen-primed B cells expressed comparable levels of cell-surface IgM and localized to the spleen after in vivo transfer. Neither of the two could proliferate in response to the mitogens. By contrast, γ-irradiated primed B cells could synthesize proteins whereas the UV treated could not. On the basis of these findings, the authors suggested that metabolic activity is necessary for activation of Ts cell by the primed B cells. Taylor et al (1989) showed that similar T cell regulation via Ts and Ta cells occurs with *P. aeruginosa* lipopolysaccharide, *S. mutans* polysaccharide and meningococcal polysaccharide and also *H. influenzae* type b polysaccharide.

Studies on the ontogeny of Ts and Ta activity (Morse et al., 1976) for the antibody response to type 3 pneumococcal polysaccharide revealed that Ts activity emerges first and is fully developed at 2 weeks of age. Ta activity, on the other hand, is minimal until week 4 and does not reach adult level until 8 -10 weeks of age. Furthermore, the immaturity of B cell subpopulations in terms of Ia density and Lyb-5 marker, would correlate with the inability of mice younger than 3-4 weeks of age to mount antibody responses to many TI antigens.

Role of spleen in immune response to polysaccharides

Studies in patients before and after splenectomy, patients with functional and anatomical asplenia and animal experiments (MacLennan et al., 1986; Amlot et al., 1985; Amlot and Hayes, 1985; Cohn et al., 1987; Wara D., 1981) have

shown that the TI-2 antigen-related immune response is specifically related to the spleen. The presence of spleen seems to be important in the primary encounter of the antigen whereas the secondary responses can also take place at sites outside the spleen. This has led to the suggestion that in the spleen, specific subsets of B cells are present or that B cells can only be triggered to respond to these types of antigens in a splenic microenvironment. In the marginal zone (MZ), where the blood leaves the arterial system into the venous sinuses, a special type of macrophages known as the marginal zone macrophages are present. By labelling the various polysaccharides with radioiodine and studying the autoradiographs, Humphrey, J.H. (1981) showed that the uncharged polysaccharides were exclusively taken up by the MZ macrophages and the acidic polysaccharides were mostly concentrated in the red pulp macrophages of mice. Kraal et al (1988), by using specific monoclonal antibodies found a much reduced MZ and thin and partly absent rings of MZ macrophages in the CBA/N mice (*xid* mice) which could explain the unresponsiveness of these mice to polysaccharides. On the basis of these results it was suggested that the MZ macrophages perhaps presented the polysaccharides to the lymphocytes. However, the findings of Kraal et al (1989) question the role of MZ macrophages in the immune response to polysaccharides. Injection of a monoclonal antibody which reacted specifically with the mouse MZ macrophages resulted in complete abrogation of the uptake of neutral polysaccharides by the cells in vivo but this did not result in an altered humoral immune response to the polysaccharide (TNP-Ficoll). Even when the MZ macrophages were completely eliminated by coupling the antibody with a toxin - gelonin, there was no alteration in the immune response against TNP-Ficoll. This would suggest that either the MZ macrophages are not involved in this kind of response or their function can be taken over by other cells.

In the MZ, a special type of B cell has been described by Bazin et al., 1982). These B cells express low levels of IgD and high levels of IgM, are intermediate in size, do not recirculate and are thought to represent a separate lineage in B cell

development restricted to the spleen. Timens et al (1989) studied the splenic MZ in infants. Whereas all other cellular compartments completed their maturation to an adult immunophenotype and morphology within the first 5 months, the MZ B cells showed different features compared to the adult MZ B cells. The infant MZ B cells had a very high percentage of cells coexpressing IgM and IgD but there was an essential lack of CD21 antigen expression. Thus the authors suggested that the hyporesponsiveness of infants to polysaccharides could probably be due to the immaturity of the splenic MZ.

Helen Braley Mullen (1990) attempted to study the properties of the antigen-presenting cell that would be required for activation of pneumococcal polysaccharide type 3 (SIII) specific suppressor T cells and contrasuppressor T (Tcs; Baker et al., 1988) cells by coupling SIII to various spleen cell subpopulations and assessing the ability of these SIII-spleen cells to activate Ts and Tcs. The results indicated that Ts and Tcs are preferentially activated when SIII is presented on distinct cell types. SIII-specific Ts were activated when SIII was coupled to anti-I-J reactive (Malley et al., 1987) plastic adherent cells, presumably macrophages and Tcs were activated when SIII was coupled to I-J negative, plastic non-adherent spleen cells.

Thus, immunity to diseases caused by invasive, encapsulated bacteria is associated with the presence of antibodies to the capsular polysaccharide. Humoral response to these bacterial polysaccharides is characterized by 1) production of predominantly IgM isotype antibody; 2) lack of a booster response; 3) delay in ontogeny; 4) IgG subclass restriction.

Immunity to mycoplasma polysaccharides

Mycoplasma mycoides subsp. *mycoides*, the causal agent of contagious bovine pleuropneumonia synthesizes a galactan that forms a slime layer. Buttery et al (1975) reported that an intravenous injection of galactan exerted specific

effects on the vascular system of the lung and on respiration which were manifested as transient apnoea, increased pulmonary arterial and decreased systemic arterial blood pressure. Necropsy revealed hemorrhages associated with alveolar ducts and vessel walls , areas of pulmonary edema, dilated airways and also some capillary thrombosis. The authors suggested that the galactan may have caused the release of biogenic amines which may have produced the effects seen. Out of the 8 calf sera that were tested, only three agglutinated galactan-coated goat red blood cells, one at a dilution of less than 1 in 80 and the other two at dilutions greater than 1 in 80. One interesting observation was that none of the calves showed any kind of reaction when they were injected for the second time with galactan using the same dose as the first time or even up to five times the first dose. The authors suggested two possible explanations for this phenomenon. First, it could be possible that galactan caused a release, to the extent of depletion, of a cell-bound mediator. The second possibility is that galactan binds for a long time with the cell receptor so that the receptor was not available for a second dose of galactan.

Several investigators have attempted to measure antibody responses to *M. dispar* antigens in the sera and lung washings from several groups of naturally or experimentally infected cattle. Scott et al (1980) examined the sera from five groups of eight calves selected from a herd reared on a beef unit in Southern England. Sera taken from these animals at about monthly intervals over a period of 200 days were examined for IgG antibody to *M. dispar* by enzyme-linked immunosorbent assay. A very small increase in the mean antibody titer of two out of the five groups was observed. Thomas et al (1982) examined paired sera , the first sample being taken at the onset of outbreaks of respiratory disease and the second about thirty days later. A fourfold increase in the IgG antibody was seen in a few of these animals. Another group of sera was examined from Ayrshire calves (Howard, 1983) . These calves had pneumonic lesions and were colonized with *M. dispar*. Although no serum antibody was detected, significant levels of IgA

antibodies to *M. dispar* were detected in the lung washings by ELISA. Howard and Gourlay (1983) reported that vaccination of gnotobiotic calves with formalin-killed *M. dispar* and oil-adjuvant by the intramuscular route followed by intratracheal administration of the killed *M. dispar* failed to protect against respiratory challenge with *M. dispar*. In contrast, three subcutaneous injections gave some evidence of protection. The investigators reasoned that the poor response to *M. dispar* could have been due to the destruction of antigens by formalin treatment and so they compared the responses to live and formalin-killed organisms in conventionally reared Ayrshire calves. They found no evidence for live mycoplasmas inducing a better serological response than killed mycoplasmas. Thus, *M. dispar* was found to be very poorly immunogenic. They also studied the serological response (IgM and IgG1 by single radial hemolysis) in conventionally reared calves of varying ages following three subcutaneous injections of *M. dispar* antigen. They found that the response was poorest in young calves and greatest in oldest calves. Almeida et al (1992) investigated the effects of encapsulated *M. dispar* and purified capsule of *M. dispar* on the activity of bovine alveolar macrophages, in vitro. They reported that the encapsulated *M. dispar* and the purified capsule, as opposed to unencapsulated *M. dispar*, did not induce and suppressed the production of tumor necrosis factor, interleukin-1 and glucose consumption by the alveolar macrophages. Thus, the very low antibody responses produced towards *M. dispar*, as reported by Howard and Gourlay (1983) could have been due to suppressive effects of *M. dispar* capsular material on the macrophages.

IMMUNE RESPONSES TO THE CAPSULAR POLYSACCHARIDE OF *MYCOPLASMA DISPAR* IN CALVES AND MICE

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Abstract --- Humoral and cell-mediated immune responses to the capsular polysaccharide (CPS) of *M. dispar* and polygalacturonic acid (pGalU - a structurally similar polysaccharide) were investigated in calves experimentally infected with *M. dispar* and in mice immunized with CPS or pGalU. Sera, tracheobronchial lavage and nasal fluids, collected before and after infection in calves, were checked for the presence of anti-CPS and anti-pGalU antibodies. The sera from mice injected with CPS or pGalU were checked for different classes of anti-CPS and anti-pGalU antibodies. Peripheral blood lymphocytes from calves and splenic lymphocytes from mice were monitored for specific proliferative responses to CPS and pGalU. At about 2 weeks post-infection, anti-CPS IgM response in serum, anti-CPS and anti-pGalU IgM and IgA response in lavage fluid and lymphocyte proliferative response was seen in the calves. Mice immunized with CPS and pGalU gave exclusively IgM responses. No secondary response was seen in mice immunized with CPS in contrast to mice immunized with pGalU. Antibodies cross-reactive with pGalU were present in the sera of CPS-immunized mice but antibodies cross-reactive with CPS were not found in pGalU-immunized mice. No significant blastogenic response was shown by mouse splenocytes to CPS or pGalU.

INTRODUCTION

Mycoplasma dispar is a capsulated mycoplasma which establishes chronic infection of the lower respiratory tract in calves resulting in local immunosuppression [1] and specific lesion production [19]. The capsular polysaccharide (CPS) is produced by the mycoplasma during natural infections [7] and in vitro, under conditions of co-culture with bovine lung fibroblasts [2]. This CPS has been shown to be involved in the suppression of several alveolar macrophage functions such as tumor necrosis factor and interleukin-1 production [1]. Thus, the CPS appears to be a major virulence factor. Acidic hydrolysis of the CPS of *M. dispar* with 4 M trifluoroacetic acid showed the presence of galacturonic acid residues [14]. In order to study the influence of galacturonic acid residues on the immunogenicty of the CPS, immune responses to polygalacturonic acid (pGalU - a linear homopolymer of galacturonic acid) were compared to those obtained against CPS.

Several attempts have been made to measure antibody responses to *M. dispar* proteinaceous antigens in naturally or experimentally infected cattle [6, 8, 17, 18] and almost none or very poor antibody responses were seen. Immune responses to the capsular antigen, however, have not been studied. This study was made to characterize the nature of the immune responses towards the CPS and to examine the extent of cross-reactivity among CPS-specific and pGalU-specific responses.

MATERIALS AND METHODS

Cattle

Three beef-type calves obtained from a *Mycoplasma dispar*-free herd were separated from the cows at birth and placed in individual isolation rooms with cedar chip bedding. They were fed limited colostrum (one pint) and a starch-free

diet which consisted of powdered skimmed milk supplemented with vitamins and minerals.

Mice

Inbred, male Balb/c mice were obtained from Taconic, Germantown, NY. They were kept in a micro-isolator (Lab Products, Maywood, NJ) on an aspen chip bedding and were fed mouse breeder sterilizable diet 7004 (Teklad, Madison, WI).

Antigens

The CPS of *Mycoplasma dispar* was produced and purified according to the method of Almeida et al [1], with minor modifications. Briefly, *M. dispar* cells were co-cultured with bovine lung fibroblast monolayers for 23 hours. After separating the mycoplasmas from the bovine cells and washing them in PBS three times at 4°C, capsule was extracted by incubating the mycoplasmas in phosphate-buffered saline at 37°C for 1 hour. The supernatant obtained after centrifugation was lyophilized. This preparation was then purified by capturing the CPS on agarose bound Ricinus communis agglutinin I or RCA_{120}, (Vector, Burlingame, CA), and eluting with 0.1 M galactose. The eluate was then passed through a Biogel P4 (Bio-Rad, Melville, NY) size exclusion column using distilled water as the running buffer. Each collected fraction was analyzed for the presence of CPS by thin-layer chromatography. A sample of 1 μl of each fraction was spotted on silica gel plate (E. Merck, Darmstadt, Germany) and was run in a solvent system of butanol, acetic acid and water in the ratio of 2:1:1. The carbohydrate spots were visualized by dipping the plate in a reagent made up of 0.5 g thymol, 0.5 g α-naphthol, 5 ml concentrated sulfuric acid and 95 ml ethanol and heating the plate for 10 minutes at 120°C. The fractions that had CPS were pooled and lyophilized. The protein and nucleic acid contamination in the purified CPS preparation was checked by measuring A_{280} and A_{260}.

Polygalacturonic acid, sodium salt was obtained from Sigma Chemical Co., St. Louis, MO.

Infection and immunization

The SD-O strain of *M. dispar* used at passage level 10 had been previously isolated from a pneumonic calf [19] and cloned twice. The calves were infected by intratracheal canulation with 10^9 cfu of *M. dispar* strain SD-O in 50 ml of sterile PBS pH 7.4 at the age of 4 weeks.

Groups of 4 Balb/c mice were injected subcutaneously one or two times, 2 weeks apart with 0.5 µg of CPS or pGalU using alum as adjuvant (Pierce, Rockford, IL). CPS and pGalU were reconstituted in saline to a concentration of 1.3 µg/ml. Alum was then added to the antigen such that the ratio of alum to alum-antigen mixture was 1:4 vol / vol. Each mouse was injected with 0.5 ml of this alum-antigen mixture. A group of 4 mice was not injected and was used as the non-injected control group. For each group of 4 mice which was injected with antigen, there was a group of 2 mice which was injected with the adjuvant alone.

Collection and processing of samples

Nasal swabs were taken from each calf twice at weekly intervals pre-infection and once after infection. The swabs were dipped in 1 ml of sterile PBS for 30 minutes and then processed for mycoplasma isolation as has been described before [11].

Nasal fluid and tracheobronchial lavage fluid were collected once before infection and twice after infection (12 and 35 post-infection). Sterile gauze tampon prepared to fit a nostril was inserted into one nostril at a time, left in place for 3 minutes, and then transferred aseptically into a chilled tube. The nasal fluid was expressed from the gauze tampon by adding 0.3 ml of PBS and centrifuging. To the fluid, 1 mM EDTA was added prior to storage at -20° C.

For the collection of tracheobronchial lavage fluid, each calf was sedated with

Rompun (Miles Laboratories, Shawnee, Kansas), 1 mg per pound body weight, injected intramuscularly. Aseptically, a mid-line skin incission was made mid-way down the neck over the trachea, and a 13 gauge cannula inserted into the trachea with the aid of a 15 gauge trocar (Tracheal wash kit, Har-Vet, Spring Valley, WI). After ascertaining that the cannula was in place (air could be aspirated readily if in place), 50 ml sterile PBS was flushed through the cannula into the tracheobronchial passage and then aspirated into a fresh syringe. Approximately 2 ml of the straw-colored fluid was recovered from each calf. The fluid was centrifuged at 700 X g for 5 minutes to remove cells. Two hundred microliters of this fluid was processed for mycoplasma isolation [11]. EDTA (1 mM) was added to the remaining fluid and stored at -20° C.

Blood for serum and peripheral lymphocytes (20 ml) was collected twice before infection and weekly after infection for 5 weeks. Sera were collected and stored at -20°C. The peripheral lymphocytes were processed for lymphocyte proliferation assay.

The sera, alveolar lavage fluid and nasal fluid were examined for the presence of anti-CPS and anti-pGalU IgG, IgM and IgA antibodies by ELISA. To measure antibodies in the lavage fluid, it was treated with an equal volume of 1:100 Sputolysin (Calbiochem, San Diego, CA) for 60 minutes at room temperature and centrifuged to remove the mucus.

The calves were euthanized 35 days post-infection. Bronchiolar swabs were taken at post-mortem using a pediatric swab with calcium alginate fiber tip (Fisher, Itasca, IL) for isolation of *M. dispar*.

Groups of mice were killed by CO_2 asphyxiation, bled by heart puncture and their spleens removed 3, 5, 9, and 12 days after the first injection, or 3, 5 and 7 days after the second injection. Prior to immunization, all mice were bled by retro-orbital puncture. The serum samples were examined for the presence of anti-CPS and anti-pGalU IgM, IgA, IgG_1, IgG_{2a}, IgG_{2b} and IgG_3 antibodies by ELISA. Spleens were processed for splenic lymphocyte proliferation assays.

ELISA

The assay was performed using 96 well, flat bottom plates (Immunolon I, Dynatech, Chantily, VA). The plates were incubated stationary with 100 μl of Ricinus Communis Agglutinin RCA_{120} (Sigma, St. Louis, MO) diluted at 500 ng/ ml in 0.1 M sodium carbonate for 14 hrs at 37°C. The plates were then washed once with ice-cold PBS and stored at 4°C until used. Just before use, the plates were washed three times with ice-cold capsule buffer (Hepes 10 mM pH 7.5; NaCl 0.15 M; $CaCl_2$ 0.1 mM). The carbohydrate, CPS or pGalU (100 ng) dissolved in 100 μl of capsule buffer, was then added to each well and incubation allowed for 1 hour at room temperature. The plates were then washed three times with ice-cold capsule buffer supplemented with 0.5% horse serum (CBHS). The coated wells were then incubated at 37°C for 1 hour with 100 μl of test sera diluted to 1:100 or 100 μl of tracheobronchial lavage fluid and nasal fluid diluted to 1:10 with CBHS. Tests were set up in duplicate. Plates were washed three times with CBHS and then incubated with 100 μl of a 1:400 dilution in CBHS of rabbit anti bovine IgG, IgM, IgA (Cappel, Malvern, PA), for 1 hour at 37°C. The plates were washed again three times with CBHS and 100 μl of horseradish peroxidase conjugated goat anti rabbit IgG (Cappel, Malvern, PA) diluted to 1:400 with CBHS was added to each well. The plates were incubated for 1 hour at 37°C and then washed three times with CBHS. In the case of murine sera, after the addition of antisera (also diluted 1:100 with CBHS), the appropriate conjugated goat anti mouse IgM, diluted to 1:200 or goat anti mouse IgA, IgG_{2a}, IgG_{2b} or IgG_3 labelled with horseradish peroxidase (Boehringer Manheim, Indianapolis, IN), all diluted to 1:400 with CBHS, was added to each well and incubation allowed for 1 hour at 37°C, followed by three washes with CBHS. The substrate solution was prepared with tablets of 5-aminosalicylic acid following manufacturer's instructions (Sigma, St. Louis, MO). After incubation for 20 minutes at room temperature, the reaction was

stopped by addition of 100 µl of 3N NaOH and the color obtained was measured spectrophotometrically at 550 nm with an automated microplate reader (Model EL310, Bio-Tek, Winooski, Vermont).

The results were expressed as mean ± SEM, where SEM was calculated as the standard deviation (SD) divided by the square root of the number of animals. The serological cut-off for a positive reaction was taken as the mean plus 2 SD of the pre-infection or pre-immunization samples. Thus, a serum was considered positive for anti-CPS IgM, anti-pGalU IgM, IgA (both anti-CPS and anti-pGalU) if the difference between the pre and post immune sera was greater than 0.020, 0.032, 0.035 O.D. units at 550nm respectively.

Peripheral blood lymphocyte proliferation assay (calves)

Lymphocyte blastogenesis (LB) was done according to the method of Roth et al [15] with a minor modification. Briefly, peripheral blood lymphocytes were collected by layering diluted blood on Histopaque 1077 (Sigma, St. Louis, MO) and centrifuging at 540 G for 40 minutes at room temperature. The hazy band was harvested and washed with Hanks balanced salt solution without calcium and magnesium (Sigma, St. Louis, MO) and finally the lymphocytes were suspended in Medium 199 (GIBCO, Gaithersburg, MD) supplemented with 1000 units/ml penicillin, 100 ng/ml kanamycin, 100 ng/ml streptomycin, 15 % fetal calf serum and 2-mercaptoethanol. The cells were then counted on a Coulter Counter (Model Z_F, Coulter Electronics, Hialeah, FL) and the cell count was adjusted to 2.5 x 10^6 cells/ml. Two hundred microlitres of the adjusted cell suspension was added in triplicate to the wells of a 96 well, flat bottom plate (Costar, Cambridge, MA) . Twenty five microlitres of antigen (1.2 mg/ml or 120 µg/ml of pGalU; 120 µg/ml or 12 µg/ml of purified CPS), ConA (20 µg/ml) or PBS was added to groups of 3 replicate wells. The plate was then incubated in a CO_2 incubator at 37°C for 5 days. Twenty two hours before harvesting, 0.25 µCi of [^{3}H] thymidine (Amersham,

Arlington Heights, IL), contained in 25µl of Medium 199 supplemented as above was added to each well. The cells were harvested onto water-prewetted glass fiber filters. These were then air dried and placed in a vial. To each vial, 5 ml of scintillation cocktail (ScintiVerse BD, Fisher, Itasca, IL) was added and the counts were done using a liquid scintillation counter (Model 1500, Packard, Downers Grove, IL). The stimulation index was calculated by dividing the mean counts per minute of triplicate antigen-stimulated cultures by mean counts per minute of unstimulated cultures.

Splenic lymphocyte proliferation assay (mice)

A cellular proliferation assay was performed with splenocytes, using the T-lymphocyte mitogen ConA (Sigma, St. Louis, MO) and the B-lymphocyte mitogen LPS from E. coli K235 (Sigma, St. Louis, MO) as the positive controls. The assay was performed as described by Lysle et al [12], with minor modifications.

On the appropriate day, the mice were killed by CO_2 asphyxiation and then were immediately bled via heart puncture. The spleen was then immediately removed and placed in a polypropylene tube containing 5 ml of RPMI-1640 medium (GIBCO, Grand Island, NY) which was supplemented with 10 mM Hepes, 2 mM glutamine and 50 µg gentamicin/ml .

A single cell suspension of each spleen was prepared by gently pressing the tissue between the ends of sterile frosted microscope slides in supplemented RPMI enriched with 10% fetal calf serum (RPMI+). The number of splenocytes was determined using a Coulter counter (Model Z_F, Coulter Electronics, Hialeah, FL) and adjusted to 5×10^6/ml.

The mitogen ConA was made to a concentration of 1.0 µg/mL and LPS to a concentration of 10.0 or 5.0 µg/mL in RPMI+. Antigens CPS and pGalU were made to a concentration of 0.5, 5.0 or 50 µg/ml in RPMI+.

One hundred microlitres of the adjusted cell suspension was added to the wells of a 96-well, flat-bottom plate (Costar, Cambridge, MA). Then, 100µl of the

antigen (CPS or pGalU) or RPMI+ (negative control) or mitogen (ConA or LPS) was added to groups of 3 replicate wells and the plates were incubated at 37°C in a humidified incubator with 5 % CO_2. The cultures were pulsed with 1 µCi [3H] thymidine (Amersham, Arlington Heights, IL) in 50 µl of RPMI+ during the last 24 hours of a 5 day incubation. The cultures were harvested onto glass fiber filters using a microharvester (Bellco Glass, Vineland, NJ). The incorporation of [3H] thymidine was determined with a liquid scintillation counter (Model 1500, Packard, Downers Grove, IL) and expressed as counts per minute from which the stimulation index was calculated.

Statistical analysis

Analysis of variance was used to assess the statistical significance of the changes in antibody levels after immunization with CPS and pGalU in mice. The level of significance for the *F* test was set at a probability of 0.001.

RESULTS

Mycoplasma isolation

No *M. dispar* was isolated from the calves prior to the experimental infection. The organism was isolated from the lavage fluid (collected at 12 and 35 days post-infection) as well as from the lungs of the infected calves at necropsy.

Antibody responses in M. dispar infected calves

In the infected calves, no serum IgA or IgG was seen in response to both CPS and pGalU (data not shown). However, there was a significant increase in the anti-CPS and anti-pGalU IgM level at 14 days post-infection (Fig. 1). The increase in the anti-pGalU IgM level was much smaller than the increase in the anti-CPS IgM level. The tracheobronchial lavage fluid did not show any IgG response and there

was a significant IgM and IgA response to both CPS and pGalU 12 days post-infection (Fig. 2). No significant IgM, IgG or IgA response was seen in the nasal fluid (data not shown).

Antibody responses of the immunized mice

The mean anti-CPS and anti-pGalU IgM level, measured as absorbance at 550 nm using ELISA, in the pre-immune sera of mice was 0.025 ± 0.00115 (mean $\pm$ SEM; n = 78 mice) and 0.051 ± 0.0020 (mean $\pm$ SEM; n = 78 mice) respectively. The mice immunized with 0.5 µg of CPS gave exclusively IgM antibody response to CPS which could be seen as early as 3 days, peaked at 9 and declined at 12 days after the first injection (statistically significant at $p < 0.001$). No antibody response was seen after the second injection (Fig. 3). Mice immunized with pGalU gave a pGalU-specific IgM response which increased from 3 days to 12 days (statistically significant at $p < 0.001$). A secondary anti-pGalU IgM response was seen beginning from 3 days and peaking at 5 days after the second pGalU injection (Fig. 4). The antibodies in the sera of mice immunized with pGalU did not show any cross-reaction with CPS while the antibodies in the sera of mice immunized with CPS did show cross-reaction with pGalU. This cross-reactive response could be seen as early as 3 days, peaked at 5 days and declined from then onwards. However, no cross-reactive antibodies were seen after the second injection of CPS (Fig. 4).

Lymphocyte proliferation

The peripheral blood lymphocytes from infected calves showed a 1.66-fold increase in the stimulation index on day 14 post-infection when stimulated with CPS at a concentration of 12 µg / ml (Fig. 5). No significant proliferative response was seen with other doses of CPS or to any dose of pGalU. No significant increase in the stimulation index of mice splenocytes was observed with any dose

of CPS or pGalU (Fig. 6).

DISCUSSION

A study to assess the immune responses produced towards the CPS of *M. dispar* in calves experimentally infected with *M. dispar* and in mice immunized with CPS showed that the immune response was typical of the response produced to polysaccharide antigens which have been characterized as thymus-independent antigens [13]. In experimentally infected calves, serum IgM response peaking at 14 days post-infection and a local humoral response (as measured in tracheobronchial lavage fluid) consisting of IgM and IgA was seen 12 days post-infection. An IgA response would normally not be expected if the antigen was exclusively polysaccharide because of limited T-cell involvement. However, in the case of infection, the host sees the polysaccharide antigen (CPS) in conjunction with *M. dispar* proteins and so it is possible that a small amount of T-cell involvement is present which enables antibody class switching from IgM to IgA. The same reason could also explain the in vitro lymphocyte proliferation seen in response to 0.3 µg of CPS (12 µg / ml). Polysaccharide-specific IgA antibodies have been shown to be produced in certain strains of mice such as C57BL/6, which were immunized with purified dextran [5]. The IgM and IgA antibody responses seen in the calves peaked at about 2 weeks post infection and then declined progressively such that by 5 weeks post-infection antibody levels were similar to pre-infection levels. Since *M. dispar* was isolated from the lung tissues and lavage fluid 5 weeks post-infection, the immune response produced appeared to be incapable of completely clearing the organism from the host. To perform these studies, calves had to be raised under conditions that minimized nonspecific antibody responses to CPS. Therefore, the calves were raised on starch-free diet in isolation rooms in order to avoid antibodies that might be formed due to exposure to other polysaccharide antigens such as starches that are present in the

hay and to CPS of other micro-organisms. They were fed limited colostrum to limit passive transfer of maternal IgG antibodies.

Mice immunized with CPS and pGalU produced exclusively IgM responses. However, no secondary response was seen in mice immunized with CPS in contrast to mice immunized with pGalU. The absence of the secondary response could be explained by: a) B-cells were tolerized with the second dose of CPS but not with pGalU, b) CPS was immunosuppressive but pGalU was not, at least not to the same extent. The difference in tolerogenicity between CPS and pGalU may be due to the differences in the molecular weights, epitope density or extent of persistence of the two polysaccharides. These factors have been found to considerably affect the tolerogenicity and immunogenicity of many polysaccharide and other thymus-independent antigens [9, 3, 4]. For example, the tolerogenicity of β2-6-linked polyfructose disappeared as its molecular weight was reduced from 6000 to 3000 [9]. The molecular weight of pGalU is 4,000 daltons in average whereas that of CPS is at least greater than 14,000 daltons [2]. The fact that the same dose of CPS (0.5 µg) was immunogenic after the first injection but tolerogenic after the second injection can be explained by the fact that the primed B cells are tolerized more easily than the potentially responsive mature B cells in unprimed mice [10].

The antibodies in the sera of mice immunized with CPS cross-reacted with pGalU but antibodies cross-reactive with CPS were not found in the sera of pGalU-immunized mice. This might indicate that among the epitopes presented by the CPS, there may be a few which are very similar to some of the epitopes of pGalU. As expected of thymus-independent antigens, no CPS-specific or pGalU-specific lymphocyte proliferation was seen in mice.

In order for the animal to be able to protect itself against infection and tissue damage, the immune responses to the capsular antigen would have to be thymus-dependent so that the host's immune system would be capable of eliciting an

anamnestic response, produce antibodies with much higher affinity to CPS and also produce IgG antibodies which can be transferred to the newborn through placenta and colostrum. Structural modifications via chemical methods and coupling of the polysaccharide with suitable protein carriers [16] are some of the approaches that could be utilized to construct thymus-dependent forms of the CPS.

In conclusion, the CPS of *M. dispar* was shown to be a T-independent antigen that elicited humoral responses cross-reactive to pGalU, a structurally similar polysaccharide. The CPS of *M. dispar* differed from pGalU as evidenced by one-way cross-reactivity of antibody responses and divergent memory responses.
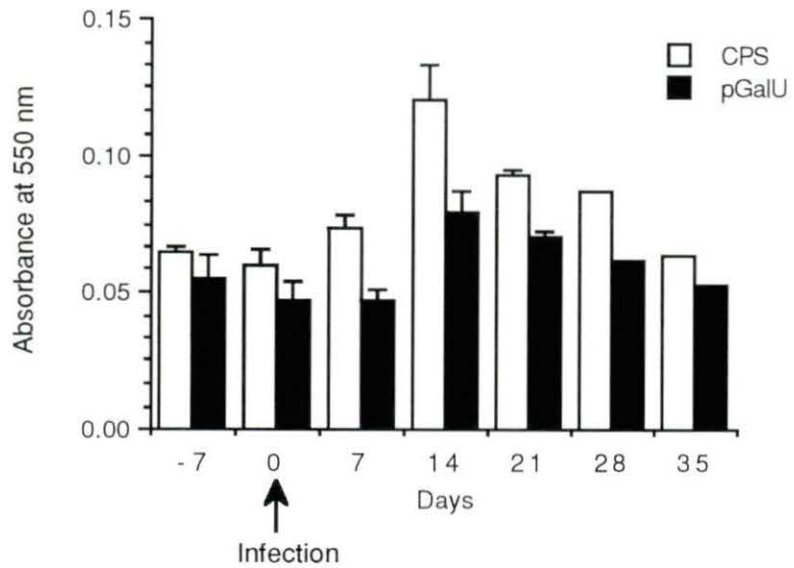
Figure 1: Serum IgM response in *M. dispar* infected calves to CPS and pGalU. Bars represent mean absorbance at 550 nm (mean ± SEM; n = 3 calves).
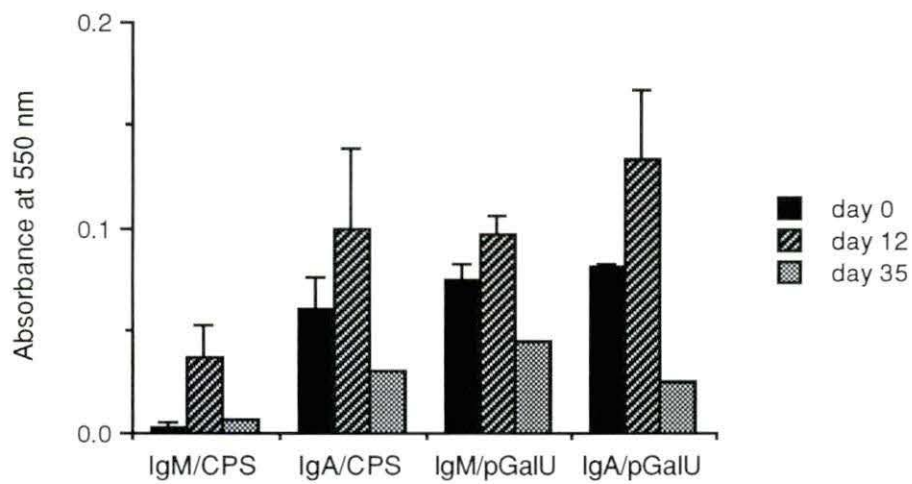
Figure 2: IgM and IgA responses in bovine alveolar lavage fluids collected on day 0, day 12 and day 35 post-infection with *M. dispar*, in response to CPS and pGalU. Bars represent the mean absorbance at 550 nm (mean ± SEM; n = 3 calves).
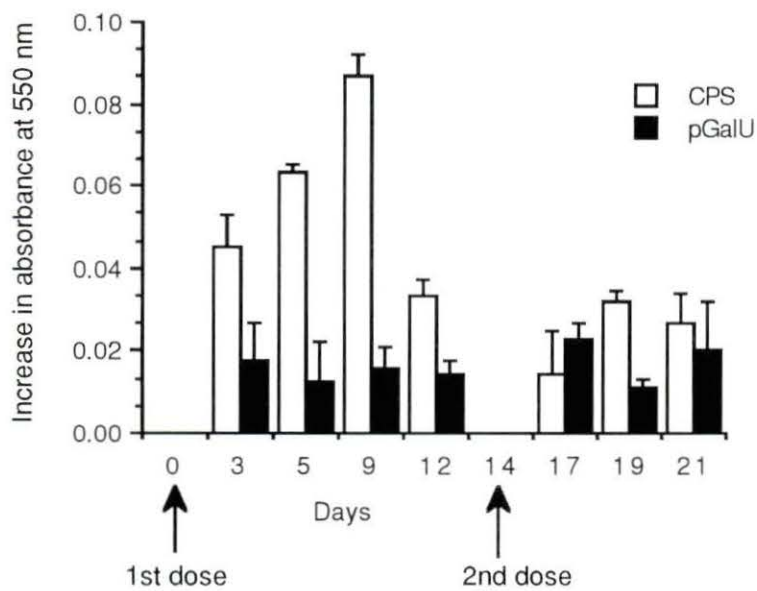
Figure 3: Serum IgM response in mice injected with CPS and pGalU tested against CPS. Bars represent the mean difference in absorbance at 550 nm calculated as post immunization serum absorbance - pre immunization serum absorbance (mean ± SEM; n = 4 mice in each group); p's < 0.001.
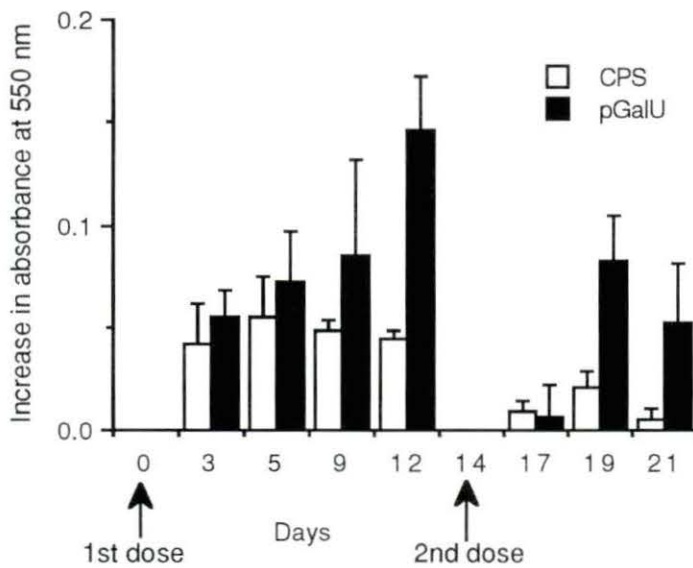
Figure 4: Serum IgM response in mice injected with CPS and pGalU tested against pGalU. Bars represent the mean difference in absorbance at 550 nm calculated as post immunization serum absorbance - pre immunization serum absorbance (mean ± SEM; n = 4 mice in each group); p's < 0.001.
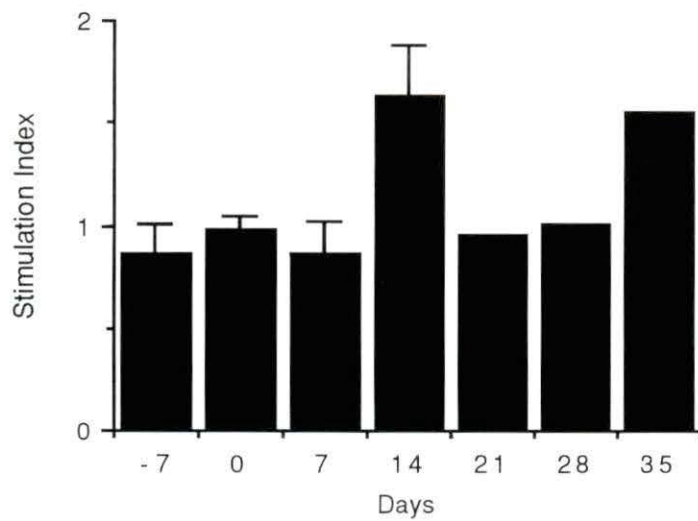
Figure 5: Proliferation of peripheral blood lymphocytes from infected calves when stimulated with 12 μg / ml CPS. Each bar represents the mean stimulation index (mean ± SEM; n = 3 calves).
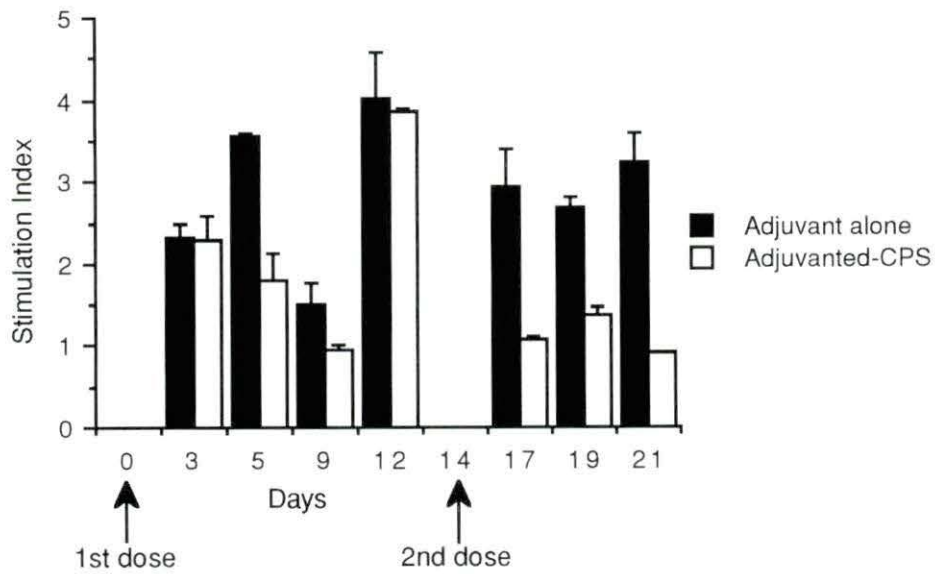
Figure 6: Proliferation of splenic lymphocytes from mice injected with 0.5 μg of CPS in response to 5 μg / ml pGalU. Response from the non-injected control mice is represented by the dotted line.

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STRUCTURAL INVESTIGATION OF THE CAPSULAR POLYSACCHARIDE OF *MYCOPLASMA DISPAR*

A paper to be submitted to *Carbohydrate Research*

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ABSTRACT

The structure of the capsular polysaccharide (CPS) of *Mycoplasma dispar* was investigated by ^{13}C-n.m.r. spectroscopy and acidic hydrolysis. A chemical shift at 175 ppm was seen in the ^{13}C-n.m.r. spectra of CPS which indicated the presence of the carboxyl groups. Acidic hydrolysis of CPS showed the presence of galacturonic acid and galactose residues. In addition, the hydrolyzate of CPS showed two more spots which could not be identified. The uronic acid content in the CPS was found to be 22 %. Based on the mass spectra of the methylated CPS, the structure of CPS was proposed to be :

-->3) - α - Gal*p* - (1-->2) - α - GalUA - (1-->3) - α - Gal*p* - (1-->

<div align="center">3</div>

<div align="center">β Gal*p* - 1</div>

INTRODUCTION

Mycoplasma dispar is a capsulated mycoplasma that produces chronic infection of the lower respiratory tract in calves resulting in local immunosuppression and specific lesion production. The capsular polysaccharide (CPS) is produced by the mycoplasma during natural infections[3] and in vitro, under conditions of co-culture with bovine lung fibroblasts[2]. This CPS has been shown to be involved in the suppression of several alveolar macrophage functions such as tumor necrosis factor and interleukin-1 production[1]. Thus, the CPS

appears to be a major virulence factor.

Although the structures of CPS from many bacteria such as *Klebsiella*, *Escherichia coli* and *Haemophilus influenzae* have been elucidated, very few studies have been focused on structures of the mycoplasma capsules. Here we report structural studies performed on the CPS of *M. dispar.*

EXPERIMENTAL

Isolation of CPS --- The CPS of *Mycoplasma dispar* was produced and purified according to the method of Almeida et al[1], with minor modifications. Briefly, *M. dispar* cells were co-cultured with bovine lung fibroblast monolayers for 23 hours. After separating the mycoplasmas from the bovine cells and washing them in PBS three times at 4°C, capsule was extracted by incubating the mycoplasmas in phosphate-buffered saline at 37°C for 1 hour. The supernatant obtained after centrifugation was lyophilized. This preparation was then purified by capturing the CPS on agarose bound Ricinus communis agglutinin I or RCA_{120}, (Vector, Burlingame, CA), washing the beads and eluting with 0.1 M galactose. The eluate was then passed through a Biogel P4 (Bio-Rad, Melville, NY) size exclusion column using distilled water as the running buffer. Each collected fraction was analyzed for the presence of CPS by thin-layer chromatography. A sample of 1 µl of each fraction was spotted on silica gel plate (E. Merck, Darmstadt, Germany) and was run in a solvent system of 1-butanol, acetic acid and water in the ratio of 2:1:1. The carbohydrate spots were visualized by dipping the plate in a reagent made up of 0.5 g thymol, 0.5 g α-naphthol, 5 ml sulfuric acid and 95 ml ethanol and heating the plate for 10 minutes at 120°C. The fractions that had CPS were pooled and lyophilized. The protein and nucleic acid contamination in the purified CPS preparation was checked by measuring A_{280} and A_{260}.

Polygalacturonic acid, sodium salt was obtained from Sigma Chemical Co.,

St. Louis, MO.

Acid hydrolysis --- Acidic hydrolysis of CPS (10 mg / ml) was performed with 4 M trifluoroacetic acid at 121° C for 1 hour. Hydrolyzate was analyzed by thin layer chromatography using 1-butanol, acetic acid and water in the ratio of 2:1:1 as the solvent.

Uronic acid content --- Uronic acids were estimated by the carbazole method as described by Knutson et al[4] with minor modifications. Sulfuric acid - borate reagent was prepared by dissolving 0.95 g of sodium tetraborate in 2.0 ml of hot water and then adding 98 ml of ice-cold concentrated sulfuric acid to it. The reagent (1.5 ml) was pipetted in each tube and kept in an ice-bath. One hundred and seventy five microliters of the sugar solution (50 - 800 µg / ml) was added to sulfuric acid - borate reagent and the tubes were again placed on ice. One hundred microliters of the carbazole solution (0.125 % in absolute ethanol) was then added. The contents of each tube were mixed well and the tubes were heated at 55° C for 30 minutes. The tubes were then allowed to sit at room temperature for 2 hours before measuring the absorbance at 530 nm.

N.m.r. spectroscopy --- The proton decoupled ^{13}C-n.m.r. spectra were recorded with a VXR-300 spectrophotometer, using D_2O as solvent. The spectra were recorded at 4° C and chemical shifts were expressed in reference to carbon disulfide.

Methylation analysis --- Methylation of CPS was performed according to the conditions of Stellner et al[5] with minor modifications. Hakomori reagent was prepared by stirring a 50 % dispersion of sodium hydride in mineral oil (3.0 g) into dry DMSO (40 ml) by a magnetic stirring bar coupled to an air driven motor in an ultrasonic bath. Dry nitrogen was blown over the surface of the mixture and the reaction was allowed to proceed at 50° C for about 4 hours by which time a clear green solution was obtained. The reagent was stored under 1 cm thick mineral oil in plastic cryovials in the freezer.

The carbohydrate, CPS (10 mg) was dissolved in water (2 ml) and 10 µg of

sodium borohydride was added. The reaction mixture was kept overnight. Excess sodium borohydride was decomposed with prewashed Dowex 50 hydrogen form (Sigma, St. Louis, MO). The sample was filtered through a membrane filter and the solution evaporated to dryness. The dried sample was dissolved in 1.0 ml of dry DMSO in a septum-stopped tube. Dry nitrogen was flushed through the tube and 0.4 ml of Hakomori reagent was added. The solution was agitated in the ultrasonic bath for 2 hours. Methyl iodide (0.4 ml) was then added dropwise and the cleared mixture was partitioned between chloroform - water (3 ml: 6 ml) three times. The chloroform layer was dried with 4 A molecular sieves (Fisher, Itasca, IL) and then evaporated to dryness. Stellner's reagent (1 ml) was added and the reaction mixture kept at 80° C for 4 hours. Water (1 ml) was then added and the reaction was allowed to occur for 4 hours at 80° C. The resulting sample was passed through a column of Dowex AG2 - x8, acetate form. The column was washed sequentially with 1.5 bed volumes each of water and methanol. The resulting solution was lyophilized. The lyophilized sample was dissolved in 1 ml of water, to which 10 mg of sodium borohydride was added. After about 2 hours, excess sodium borohydride was decomposed with Dowex 50 hydrogen form. After filtration through a membrane filter, the solution was evaporated to dryness. The residue was treated with acetic anhydride-pyridine; 1:1 (4 ml) for 30 minutes at 60° C. The mixture was then partitioned between chloroform - water (3 ml : 6 ml) three times. The chloroform layer was dried with 4 A molecular sieves and the sample was concentrated by blowing dry nitrogen over it. The acetylated sample was analyzed by g.c-m.s using a DB-5 column.

RESULTS

Sugar analysis --- The uronic acid content in the CPS of *M. dispar* was found to be 22 % by carbazole analysis. Acidic hydrolysis of CPS for 1 hour with 4 M TFA showed 4 spots on the TLC (Table 1). Two out of the 4 spots could be identified as D-galacturonic acid and D-galactose and the other two could not be

identified.

N.m.r. spectra --- ^{13}C n.m.r. spectra of CPS showed 4 clear peaks (Fig. 1). One was at 174.90 for the C=O of the carboxyl group and 3 peaks at 50.5, 56.6 and 59.2 ppm for C-2, C-3 and C-4 of galactose respectively. In addition, one very small peak at 105.1 ppm corresponding to the anomeric carbon atom and another small peak at 61.0 ppm for C-5 was seen.

Methylation analysis --- The mass spectra of the methylated CPS is shown in figure 2. The peaks obtained could be explained when the CPS was proposed to be a tetrasaccharide repeat consisting of three neutral sugar residues (all galactose residues) and one charged galacturonic acid residue. The galacturonic acid residue and two galactose residues form the backbone and one galactose residue forms the side chain (Fig. 3)

The various possible fragments that would be obtained from the proposed structure when subjected to the methylation process which could account for the peaks seen in the mass spectra of the methylated CPS are shown in figure 4.

DISCUSSION

The immunogenicity and the immunological specificity of polysaccharides is dictated by their structures. Hence, in order to understand the immunology of polysaccharides and to develop effective prophylactic vaccines against encapsulated microorganisms, it is imperative that the structure of the polysaccharide capsule be elucidated. Structural investigation of the CPS of *M. dispar* revealed that the CPS consisted at least of galacturonic acid and galactose residues. Since the uronic acid content was found to be 22 % acidic sugar, it may indicate that out of every 4 residues in the CPS, one of them is galacturonic acid. We have demonstrated that there is considerable cross-reactivity among anti-CPS and anti-polygalacturonic acid antibodies[5]. This may indicate that galacturonic acid may be the immunodominant sugar.

Hydrolysis of the CPS with TFA showed 4 spots, out of which 2 could not be

identified. Both these spots had higher R_f values than galactose or galacturonic acid. These could be other hexoses or they could be 4-C or 5-C degradation products of galacturonic acid.

Direct chemical analyses of few mycoplasma capsules has been reported. The CPS of *M. mycoides* subsp. *mycoides* is a galactan which is made up of a repeating unit of β-D-galactofuranosyl-(1-->6)-D-galactopyranose[7]. The predominant structural unit of the glucan from the *Mycoplasma* sp. bovine arthritis strain is β-D-glucopyranosyl-(1-->2)-D-glucopyranose[8]. The composition of the polysaccharide obtained from F-38 strain of mycoplasma (etiologic agent of contagious caprine pleuropneumonia) was determined to be a mixture of glucose, galactose, mannose, fucose, glucosamine and galactosamine in approximately equal quantities[9]. In all these cases, the purification of the capsular material was accomplished by using hot phenol extraction procedures followed by ethanol precipitation or ion-exchange chromatography. It has been shown that hot phenol extraction causes the removal of lipoglycans present in the mycoplasma membrane[10]. The results could, therefore, have been affected by the presence of such impurities.

D-Galacturonic acid residues have been shown to be present in a number of bacterial capsules such as those of *Klebsiella* serotype K 49[11] and *Streptococcus pneumoniae.* This report shows that the CPS of *M. dispar* possesses such galacturonic acid residues.

TABLE I: R_f values of the components obtained by acid hydrolysis of CPS and pGalU.

Compound	Component	R_f
CPS	Galacturonic acid	0.341
	Galactose	0.488
	Unidentified	0.682
	Unidentified	0.804
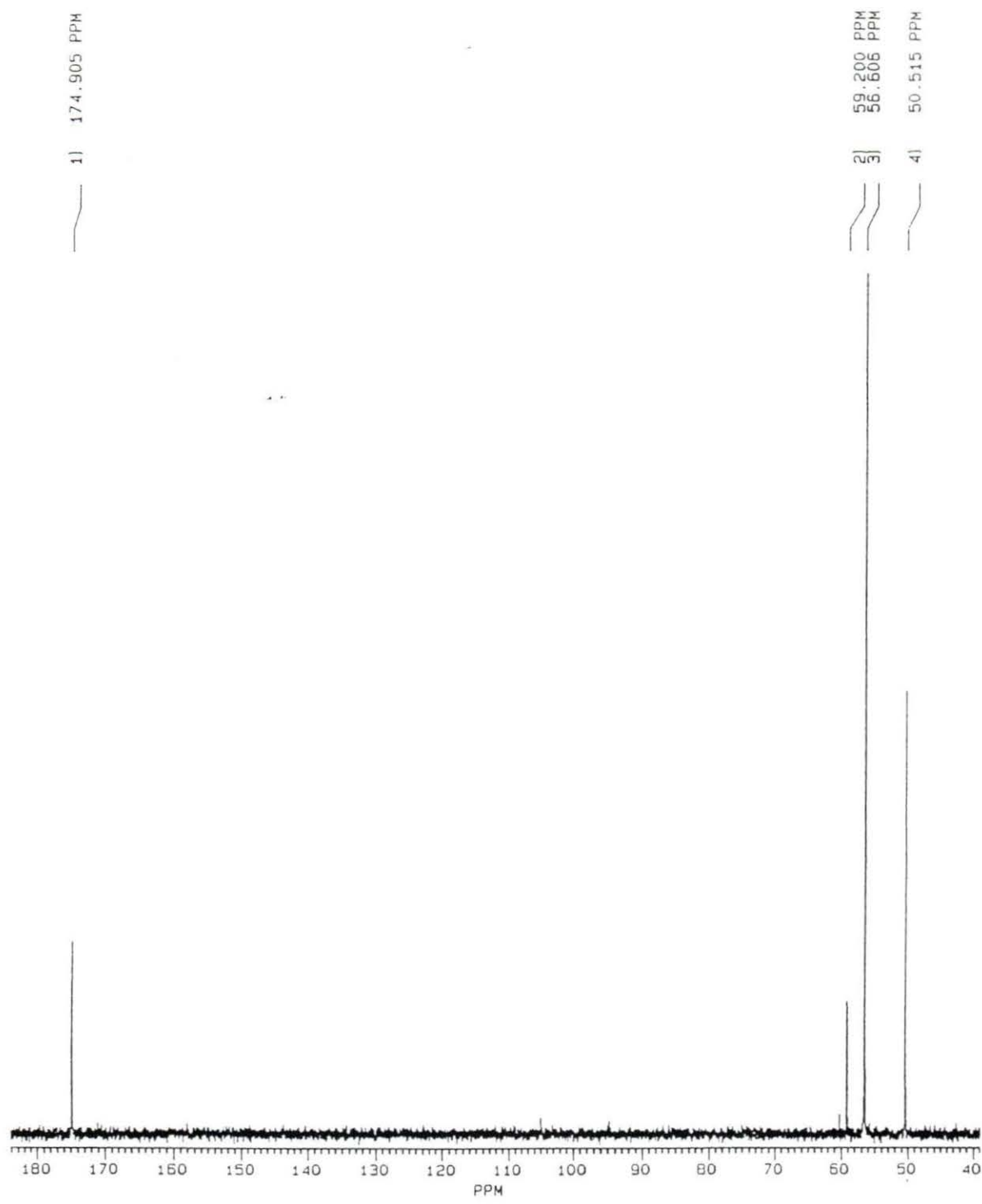
Figure 1: ^{13}C NMR of CPS
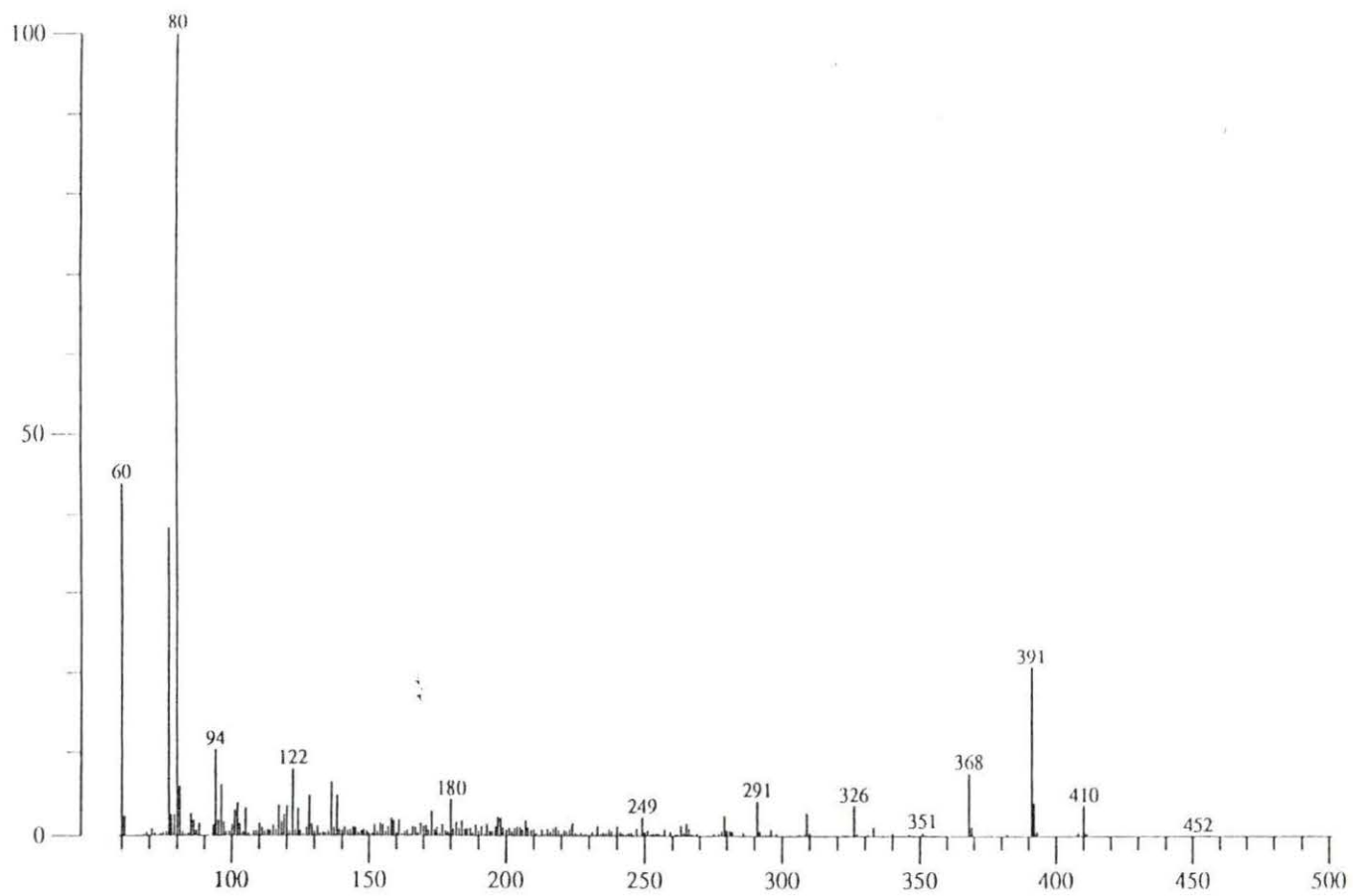
Figure 2: Mass spectra of methylated CPS
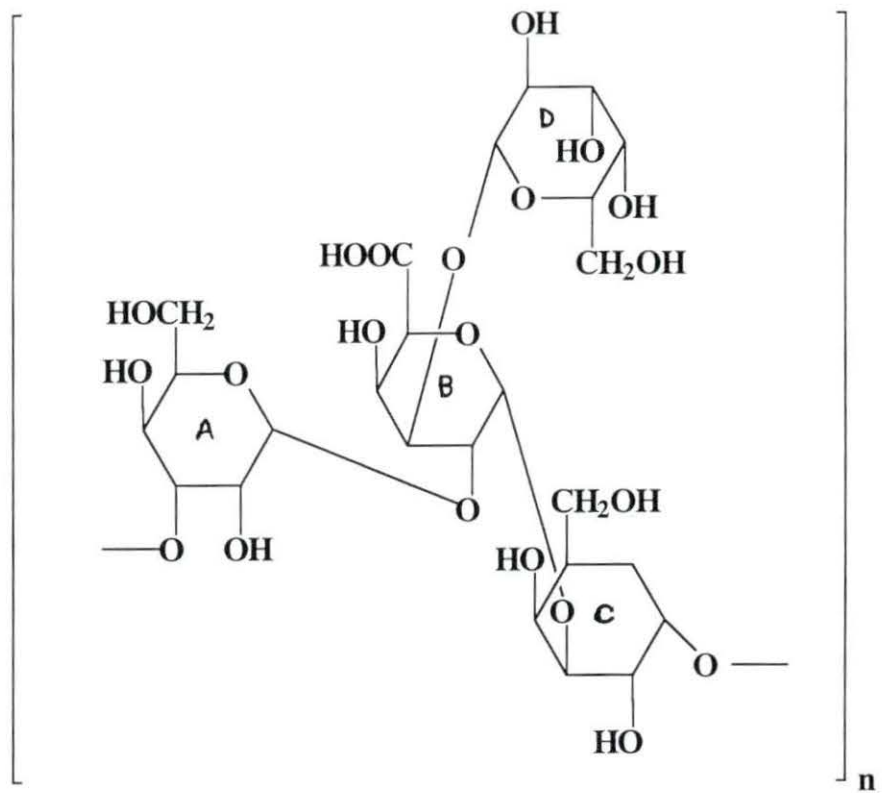
Figure 3: Proposed structure of CPS

A)

```
        H₂C–OAc
           |                    73ᵃ
        H–C–OMe             277ᵇ
           |              117
        AcO–C–H           233
           |                    189
        MeO–C–H           161
           |                         233
        H–C–OAc           117
           |                              305
        H₂C–OMe           45
```
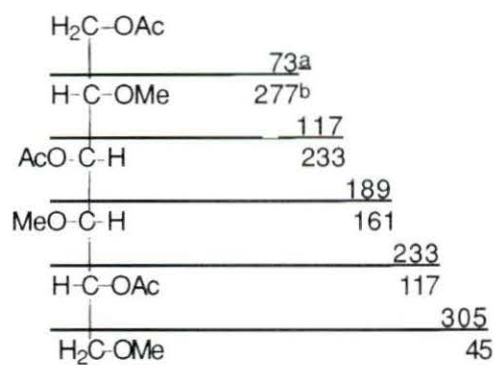
m/e = 350

m/e 161 - m/e 32 (CH₃OH) --> m/e 129 - m/e 42 (C₂H₂O) --> m/e 87

m/e 232 - m/e 60 (CH₃COOH) --> m/e 173

m/e 277 - m/e 32 --> m/e 245 - m/e 42 --> m/e 203

B)

```
        H₂C–OAc
           |              73
        H–C–OAc           319
           |                    145
        AcO–C–H           247
           |                         217
        MeO–C–H           175
           |                              261
        H–C–OAc           131
           |                                   333
        H₂C–OMe           59
```

m/e = 392

m/e 175 - m/e 32 --> m/e 143 - m/e 42 --> m/e 101

m/e 247 - m/e 60 --> m/e 187

m/e 319 - m/e 60 --> m/e 259

Figure 4: Fragmentation of methylated products obtained from residues A and B as indicated in Fig. 3
   ᵃ The figures are the m/e values of the upper fragment
   ᵇ The figures are the m/e values of the remaining fragment

C)

```
                H₂C-OAc
         _____|_____73a___
                H-C-OMe          277b
         _____|_____117_____
                AcO-C-H                  232
         _____|_____188_____
                MeO-C-H                    161
         _____|_____233_____
                H-C-OAc                      117
         _____|_____45___
                H₂C-OMe                      305
```
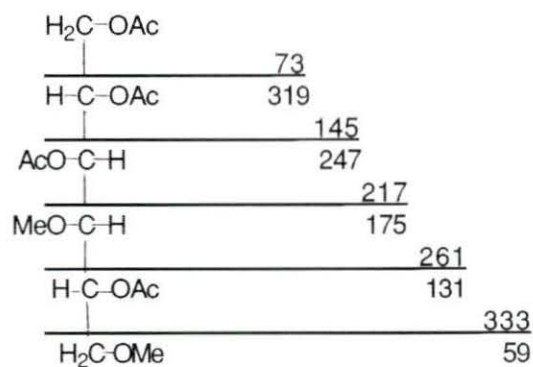
m/e = 350

m/e 161 - m/e 32 --> m/e 129 - m/e 42 --> m/e 87

m/e 232 - m/e 60 --> m/e 172

m/e 277 - m/e 32 --> m/e 245 - m/e 42 --> m/e 203

D)

```
                H₂C-OAc
         _____|_____73a___
                H-C-OMe          249b
         _____|_____117_____
                MeO-C-H                  205
         _____|_____161_____
                MeO-C-H                    161
         _____|_____205_____
                H-C-OAc                      117
         _____|_____277___
                H₂C-OMe                      45
```

m/e = 322

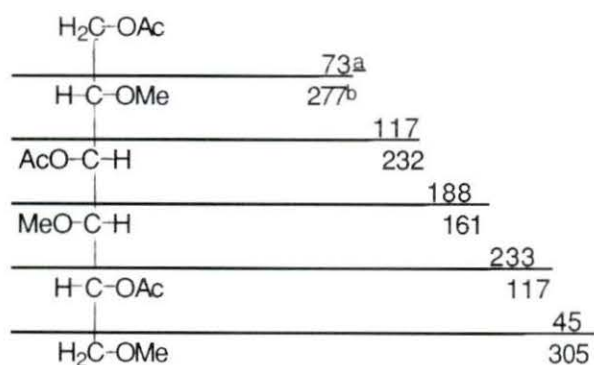m/e 161 - m/e 32 --> m/e 129 - m/e 42 --> m/e 87

m/e 205 - m/e 32 --> m/e 173

m/e 249 - m/e 32 --> m/e 217

Figure 5: Fragmentation of methylated products obtained from residues C and D
as indicated in Fig. 3
[a]The figures given are the m/e values of the upper fragment
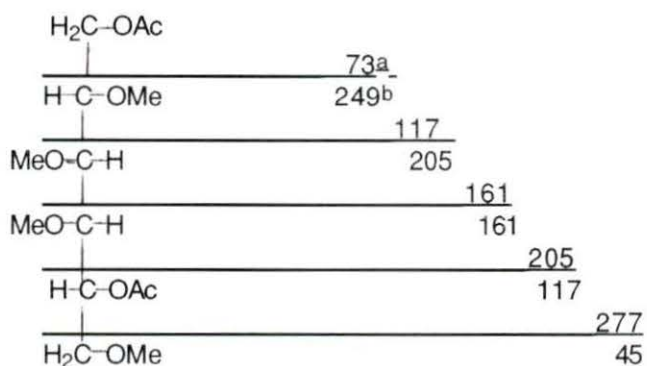[b]The figures given are the m/e values of the remaining fragment

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GENERAL SUMMARY

Three research goals were outlined in the Introduction section of this thesis. The first, to characterize the immune responses towards pGalU and to the purified capsule of *M. dispar* in mice and the second, to characterize the immune responses to CPS and pGalU in *M. dispar* infected cattle have been achieved. The first goal was aimed at understanding the antigenic nature of the purified CPS. Generally, polysaccharides have been classified as thymus-independent type 2 antigens. The results obtained from the studies done in mice strongly indicate that the purified CPS acts as a typical thymus-independent antigen. It was however not possible to state the particular type to which it belongs i.e. whether type 1 or 2 thymus-independent antigen because that would require that the studies be done in neonatal and xid mice. The studies done in *M. dispar* infected cattle support the thymus-independent antigenic nature of CPS. These studies were necessary in order to make sure that the association of CPS with *M. dispar* did not endow unique properties to the CPS and also that the process of extraction and purification of CPS did not destroy or alter the antigenicity or immunogenicity of the CPS.

These two goals were also aimed at studying the cross-reactivity among the antibodies directed towards CPS and pGalU. Studies in mice and in calves indicate considerable cross-reactivity which means that there is potential for use of inexpensive, commercially available pGalU instead of CPS in vaccines based on the polysaccharide antigen.

The third research goal was to chemically characterize the structure of CPS. This goal has been partly achieved. One of the major hurdles in accomplishing this goal was the lack of availability of large amounts of purified CPS. However, the data obtained does indicate similarity between CPS and pGalU acid. The presence of 22 % acidic sugar may indicate that among every 4 residues there is one galacturonic acid residue. The considerable cross-reactivity seen among anti-

CPS and anti-pGalU antibodies may also indicate that the galacturonic acid residue is the immunodominant sugar.

Overall, this work provided good evidence for the thymus-independent nature of the CPS of *M. dispar* which may explain the ability of *M. dispar* to establish chronic colonization of the lower respiratory tract in calves. Structural studies performed revealed that the CPS contained galacturonic acid and galactose residues. The presence of the galacturonic acid residues formed the basis of the cross-reactivity seen among the CPS-specific and pGalU-specific antibodies.

Since, the thymus-independent responses seen in *M. dispar* infected calves appeared to be incapable of clearing the organism from the host it is evident that forms of CPS that induce thymus-dependent responses will have to be constructed. Since there is structural similarity between polygalacturonic acid and CPS, it may be possible to use chemically modified forms of pGalU or pGalU-protein conjugates for inducing immunity in calves.

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APPENDIX. STUDY OF IMMUNE RESPONSES IN CATTLE IMMUNIZED WITH POLYGALACTURONIC ACID

INTRODUCTION

This appendix summarizes an attempt to study the antibody responses towards the capsular polysaccharide (CPS) of *M. dispar* and polygalacturonic acid (pGalU) in cattle immunized with pGalU.

There is preliminary evidence that the capsule of *M. dispar* is similar in chemical composition to polygalacturonic acid which is found in citrus rinds and other plant tissues.To determine whether polygalacturonic acid could replace CPS as the antigen in vaccines it was necessary to characterize the imune response to polygalacturonic acid in cattle and examine whether the antibodies produced towards polygalacturonic acid would cross-react with CPS.

MATERIALS AND METHODS

Cattle

Thirteen cross-bred beef type steers,17 to 15 months old, were injected subcutaneously with 2.5µg of polygalcturonic acid in 2 ml of saline to which 1 ml of Alum Inject (Pierce, Rockford, IL) was added and test bled for serum samples according to the following schedule:

Day	Sampling
d0	Test bled and 1st vaccination
d14	Test bled
d30	Test bled and 2nd vaccination
d35	Test bled

Serums were collected and stored at -20C until tested for the presence of IgG and IgM antibodies to polygalcturonic acid and CPS using ELISA.

ELISA

The assay was performed as has been described previously in paper 1. The controls used in the test were:

1. Substrate control : RCA_{120} + CPS + substrate

2. Conjugate control : RCA_{120} + CPS + horseradish peroxidase conjugated goat anti rabbit IgG + substrate

3. Anti-class control : RCA_{120} + CPS + rabbit anti bovine IgG or IgM + horseradish peroxidase conjugated goat anti rabbit IgG + substrate

Statistical analysis

Analysis of variance (Table 5.0) was done to see whether there were any significant differences in the antibody levels on different days.

RESULTS

Both anti-CPS (Table 1 and 2) and anti-pGalU antibodies (Table 3 and 4) could be detected in the sera of the steers vaccinated with pGalU. The anti-pGalU IgM antibody values were higher than anti-pGalU IgG, anti-CPS IgM and anti-CPS IgG values. Table 5 shows the means and the standard errors of means of the anti-CPS and anti-pGalU antibody values obtained from the sera of the thirteen steers.

After immunization with pGalU, no IgG response was seen with either CPS or pGalU. However, significant changes in the anti-CPs anti-pGalU levels were seen after immunization. The anti-CPS level decreased significantly at 30 days post-immunization. After the second dose of pGalU, the anti-CPS antibody level increased and reached the pre-immunization level. The anti-pGalU IgM level

decreased significantly at day 14 post-immunization, then increased such that by day 30 the antibody level was same as the pre-immunization level and then decreased again after the second immunization such that the IgM level was similar to that on day 14 post-immunization.

DISCUSSION

Carbohydrates are considered as thymus-independent antigens which means that they induce primarily a humoral response and the antibody isotype is predominantly IgM. However, no increase in the anti-CPs or anti-pGalU IgM level over the pre-immunization was seen. The anti-pGalU level declined 14 days post-immunization, climbed back by day 30 and then declined again after the second dose of pGalU. This may indicate a phenomenon of antibody-mediated suppression. The first dose of antigen (pGalU) resulted in the clearance of some already present anti-polysaccharide antibodies. This decrease in the antibody level enabled the antigen to activate specific B cells resulting in the production of specific antibodies. After the second injection, the antigen again cleared the preexisting antibodies resulting in a decrease of anti-pGalU antibodies.

From the above results it is evident that the steers had high levels of preexisting levels of antibodies to pGalU and also to the CPS of *M. dispar* (although these steers came from a *M. dispar* free herd). This reactivity to the CPS and to pGalU may have stemmed from two possible sources: antibodies may have been produced in response to the various carbohydrates in the feed or due to the exposure to the CPS of other bacteria. It becomes clear that t study the immune responses to pGalU or to the *M. dispar* CPS, calves will have to be raised in isolation so that they do not get exposed to other encapsulated bacteria and should preferably be fed a non - starch diet.

Table 1: Results of antoglobulin-ELISA for detecting anti-CPS IgG antibodies in sera of the thirteen steers before and following vaccination with pGalU

Day	Steer # 432	434	439	440	441	442	443	444	445	446	447	450	452
0	.206a	.203	.205	.204	.195	.241	.237	.225	.224	.210	.209	.207	.205
14	.202	.193	.197	.201	.199	.244	.237	.228	.226	.220	.211	.207	.240
30	.201	.205	.209	.202	.197	.219	.233	.230	.223	.225	.203	.204	.238
35	.184	.200	.208	.196	.204	.228	.232	.229	.226	.235	.208	.208	.233

aThe figures given are optical density at 550 nm.

Table 2: Results of antiglobulin-ELISA for detecting anti-CPS IgM antibodies in sera of the thirteen steers before and following vaccination with pGalU

Day	Steer # 432	434	439	440	441	442	443	444	445	446	447	450	452
0	.246[a]	.244	.216	.251	.196	.258	.269	.234	.180	.227	.249	.252	.278
14	.214	.251	.219	.228	.197	.269	.238	.186	.209	.235	.246	.260	.262
30	.224	.265	.230	.230	.172	.227	.251	.166	.180	.203	.237	.265	.241
35	.227	.248	.255	.245	.216	.229	.239	.188	.180	.230	.237	.265	.241

[a]The figures given are optical density at 550 nm

Table 3:　　　　Results of antiglobulin-ELISA for detecting anti-pGalU IgG antibodies in sera of the thirteen steers before and following vaccination with pGalU

Day	Steer # 432	434	439	440	441	442	443	444	445	446	447	450	452
0	.179[a]	.187	.196	.197	.207	.217	.211	.190	.233	.261	.227	.194	.184
14	.198	.199	.198	.205	.192	.208	.205	.201	.222	.221	.229	.225	.190
30	.198	.199	.186	.195	.214	.211	.208	.169	.223	.227	.189	.217	.174
35	.191	.208	.201	.189	.204	.215	.207	.194	.218	.226	.219	.215	.188

[a]The figures given are optical density at 550 nm

Table 4:   Results of antiglobulin-ELISA for detecting anti-pGalU IgM antibodies in sera of the thirteen steers before and following vaccination with pGalU

Day	Steer # 432	434	439	440	441	442	443	444	445	446	447	450	452
0	.301[a]	.302	.296	.308	.317	.313	.315	.297	.260	.291	.294	.304	.289
14	.287	.306	.294	.301	.285	.295	.302	.244	.202	.286	.276	.285	.292
30	.304	.293	.306	.307	.305	.305	.315	.267	.253	.299	.273	.290	.290
35	.278	.293	.307	.294	.279	.301	.287	.266	.187	.293	.291	.278	.288

[a]The figures given are optical density at 550 nm

Table 5:   Results of antiglobulin-ELISA for detecting anti-CPS IgG, IgM and anti-pGalU IgG, IgM in sera of thirteen steers

Antibodies	Days 0	14	30	35
Anti-CPS IgG	a.213 ± .0036	.216 ± .0047	.215 ± .0036	.215 ± .0044
Anti-CPS IgM	.238 ± .0072	.232 ± .0069	.220 ± .0083	.231 ± .0063
Anti-pGalU IgG	.206 ± .0061	.207 ± .0033	.201 ± .0047	.206 ± .0033
Anti-pGalU IgM	.299 ± .0061	.281 ± .0074	.293 ± .0049	.280 ± .0080

aThe figures given are the means of the values ± the standard error mean of the optical density at 550 nm of the sera of the thirteen steers when tested by antiglobulin-ELISA

Table 6.1:   Analysis of variance table of anti-CPS IgG antibodies

Source	[a]DF	Anova [b]SS	F value	Pr > F
Cow	12	0.00969092	13.84	0.0001
Day	3	0.00004492	0.26	0.8561

[a]DF = degrees of freedom
[b]SS = sum of squares

Table 6.2:   Analysis of variance table of anti-CPS IgM antibodies

Source	[a]DF	Anova [b]SS	F value	Pr > F
Cow	12	0.02830658	10.59	0.0001
Day	3	0.00209883	3.14	0.0370

[a]DF = degrees of freedom
[b]SS = sum of squares

Table 6.3:   Analysis of variance table of anti-pGalU IgM antibodies

Source	[a]DF	Anova [b]SS	F value	Pr > F
Cow	12	0.02169892	12.10	0.0001
Day	3	0.00328360	7.33	0.0006

[a]DF = degrees of freedom
[b]SS = sum of squares

Table 6.4:   Analysis of variance table of anti-pGalU IgG antibodies

Source	[a]DF	Anova [b]SS	F value	Pr > F
Cow	12	0.01018073	7.41	0.0001
Day	3	0.00032560	0.95	0.4278

[a]DF = degrees of freedom
[b]SS = sum of squares

Table 7:   Duncan multiple range test for anti-CPS IgM antibodies

Duncan Grouping	Mean	Day
A	0.238462	0
A	0.231846	14
A	0.230692	35
B	**0.220692**	**30**