

THE PRODUCTION AND
FUNCTIONAL ^{ASSESSMENT} ~~CHARACTERIZATION~~
OF ~~MUCOSALLY DERIVED~~ IGA
MONOCLONAL ANTIBODIES.

by

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ABSTRACT .

This thesis describes the production of IgA monoclonal antibodies using mucosal immunization and their functional assessment with in vitro assay systems.

Rat monoclonal antibodies of IgA, IgM and IgG1 isotype, directed against an auxotrophic mutant strain of Salmonella typhimurium SL3261 BRD026 (SL/BR) were produced by Peyer's patch immunization and the use of either mesenteric lymph nodes or efferent mesenteric lymph as a source of primed lymphocytes.

Thirty-two hybridoma cell-lines secreting monoclonal antibodies specific for Salmonella typhimurium SL3261 BRD026 (SL/BR) were cloned. These Mabs were characterized for specificity and a panel of 18 specific for lipopolysaccharide (13 IgA, 4 IgM and 1 IgG1) were assessed for their ability to prevent adherence of the immunizing strain to Madin-Darby canine kidney (MDCK) cell monolayers. All of the Mabs were capable of inhibiting adherence except for the IgG1 antibody.

Studies on the physicochemical nature of treated and untreated Salmonella typhimurium SL3261 BRD026 (SL/BR) were carried out using Octan-1-ol partitioning, hydrophobic interaction chromatography and partitioning in aqueous two-phase polymer systems.

No correlation was found between the effects of monoclonal antibody treatment on bacterial cell-surface hydrophobicity and adherence to Madin-Darby canine kidney (MDCK) cell monolayers. However, treatment with both IgA

and IgM Mabs increased the net negative surface charge of the bacteria and this correlated with the degree of inhibition of adherence to Madin-Darby canine kidney (MDCK) cell monolayers.

Multiple molecular forms were seen with 9 of the 13 IgA monoclonal antibodies cloned. These molecular forms were isolated for 2 of the monoclonal antibodies concerned and their effects on adherence to Madin-Darby canine kidney (MDCK) cell monolayers and the physicochemical nature of Salmonella typhimurium SL3261 BRD026 (SL/BR) were investigated.

All molecular forms inhibited adherence to Madin-Darby canine kidney (MDCK) cell monolayers and produced the same degree of decrease in the bacterial cell surface hydrophobicity. However, the forms varied in their effects on the bacterial cell surface charge.

Monoclonal antibodies of IgA, IgM and IgG1 isotypes, previously prepared by other workers, were also used to assess the use of a chemiluminescence assay for the comparison of the opsonic capabilities of the isotype.

The chemiluminescence assay parameters were found to be sub-optimal for IgA monoclonal antibodies compared with IgG1.

The data presented shows that mucosal immunization and use of mucosal tissue for monoclonal antibody production is an effective method for the production of large numbers of IgA monoclonal antibodies.

The physicochemical effects of treatment with these monoclonal antibodies is of importance in the prevention of adherence in vitro.

Comparison of IgA monoclonal antibodies with other isotypes in conventional assays designed for systemic immune components can result in inaccurate assessments if the parameters are not optimized for IgA.

DEDICATION .

For my wife, Abigail and our daughter, Hannah.

"Sugar and spice
And all things nice,
That's what little girls are made of."

Oxford Dictionary of Nursery Rhymes.

And my parents, Ann and Christopher Joseph Joyce.

"Patience is a virtue have if you can,
seldom in a woman and never in a man."

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"What is now proved was once only imagin'd"

William Blake, the Marriage of Heaven and Hell

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Abbreviations.

Chemiluminescence	CL
Colony forming unit	CFU
Foetal calf serum	FCS
Gastro-intestinal associated lymphoid tissue	GALT
Immune Rabbit serum	IRS
Intra-epithelial lymphocytes	IEL
Lipopolysaccharide	LPS
Madin-Darby canine kidney cells	MDCK
Maximal growth medium	MGM
Mesenteric lymph node	MLN
Monoclonal antibody	MAb
Mucosa associated lymphoid tissue	MALT
Peyer's patch	PP
Phosphate buffered saline	PBS
Polymorphonuclear leucocytes	PMN
<u>Salmonella typhimurium</u> SL3261	SL
<u>Salmonella typhimurium</u> SL3261 BRD026	SL/BR
Secretory component	SC
Secretory IgA	SIgA
Secretory IgM	SIgM

1 . INTRODUCTION .

1.1 MUCOSAL IMMUNITY.

The total area of mucosal surfaces in humans has been estimated at in excess of 400 m² and so presents an enormous area over which antigens, both environmental and microbial, are first seen by the immune system (Kilian et al., 1988). These antigens can include many infectious agents, both bacterial, rickettsial and viral. Some infectious agents initially gain entry via mucosal surfaces without causing any mucosal pathology and can subsequently give rise to systemic disease (Davis et al., 1980; Mandell et al., 1985). There are others that either cause pathological effects at the mucosal surface such as cholera or that cause both mucosal and systemic disease such as salmonellosis (Wells et al., 1988). It is apparent that the immune mechanisms associated with the mucosal surfaces are of primary importance in the prevention of many serious infectious diseases. Allergens both from food and the environment increase the antigenic load found at the mucosal surface. Local immune responses which reduce the level of absorption of these antigens and aid in producing systemic immune hyporesponsiveness are important if allergic reactions are to be avoided (Challacombe and Tomasi, 1980). Again it can be seen that the role of the immune mechanisms at the mucosal surface in producing these responses would be of great interest

in the understanding of many harmful allergenic conditions.

The idea of local immunity at mucosal surfaces, independent of systemic immunity, was first postulated by Besredka (1919) based on the results of experimental studies of oral infections with toxin-producing enterobacteria and skin infections with Bacillus anthracis. These studies were followed in 1927 by a classic paper, "Local Immunization", (Besredka, 1927) in which he summarized the evidence available at that time to suggest that a regional immune state could exist without significant participation from serum antibody.

The concept was extended by later workers, particularly Burrows and Fazekas de Groth who worked, respectively, on experimental cholera in guinea pigs and influenza virus infection in mice.

Burrows and Havens (1948) showed that resistance to cholera in the guinea pig model was dependant on the titres of "coproantibody" as he termed the antibodies present in gastrointestinal secretions. The pathology caused by cholera at the mucosal surface occurred without invasion of the host and so it was reasonable to assume that the effective immunity was derived from antibody at the mucosal surface. Serum antibody titres were found to develop after immunity was established and as such were ruled out as the origin of the coproantibody at least by simple transudation.

Fazekas de Groth and Donnelly (1950) showed that resistance to influenza virus in the respiratory tract of

mice seemed to be directly related to the level of specific antibody in the fluids bathing the mucous membranes. Antibody of significant titre prevented the implantation of influenza virus and so led to immunity.

In spite of this early work the concept of local mucosal immunity was not fully appreciated until the early 1960's and the description of IgA secretory antibodies in 1961 by Hanson (Hanson, 1961). This finally led to the idea that there existed a "secretory immune system" with unique properties which was located at the mucosal surface and which under normal circumstances could function quite independently of the "systemic immune system" which was responsible for the production of circulating antibody (Tomasi et al., 1965).

1.1.1. The common mucosal immune system.

Specific mucosal immunity is now recognized as being provided by mucosa associated lymphoid tissue (MALT), a collection of lymphoid tissues found in the gastrointestinal, bronchial and genito-urinary tracts and glandular tissue throughout the body. MALT is also believed to form a common mucosal immune system where antigenic stimulation at one mucosal surface leads to a subsequent dissemination of cells which home to distant mucosal sites transferring specific immunity throughout the mucosal tissues of the body. The concept of a common mucosal immune system linked by antigen-specific emigrating IgA precursor cells has been supported by antibody induction studies (Goldblum et al., 1975), cell

migration experiments and morphologic studies (Bienenstock et al., 1983).

Early cellular analysis of Peyer's patches (gastrointestinal lymphoid organs forming part of MALT) showed them as enriched sources of IgA precursor cells (Craig and Cebra, 1971) which on antigen stimulation migrated to the mesenteric lymph nodes. There they matured into antibody-secreting plasma cells and then seeded themselves in the lamina propria and bronchial associated mucosal tissue (Rudzik et al., 1975). Further studies revealed that mesenteric lymph node blast cells capable of secreting IgA also home to mammary glands (Roux et al., 1977), genital mucosa (Bienenstock and Befus, 1980), salivary glands (Jackson et al., 1981) and ocular tissue (Mestecky et al., 1978). Recent work has also identified the migration of T-cells (Bienenstock et al., 1983), the role of antigen-specific memory cells (Pierce and Cray, 1982) and the possible induction of IgG antibodies (McDermott and Bienenstock, 1979) as aspects of the common mucosal immune system.

The mechanisms controlling homing of IgA precursor cells to distant mucosal sites are as yet unclear (Weisz-Carrington et al., 1979). The homing phenomenon has been shown to occur even without antigen stimulation (Roux et al., 1977) but requires the presence of T helper cells specific for IgA (Bienenstock et al., 1983). There is also some evidence which supports a role in homing for membrane glycoproteins and/or glycolipids on migrating lymphocytes by interaction with endothelial cells (Hooghe

and Pink, 1985) but further work will need to be carried before the system is fully understood.

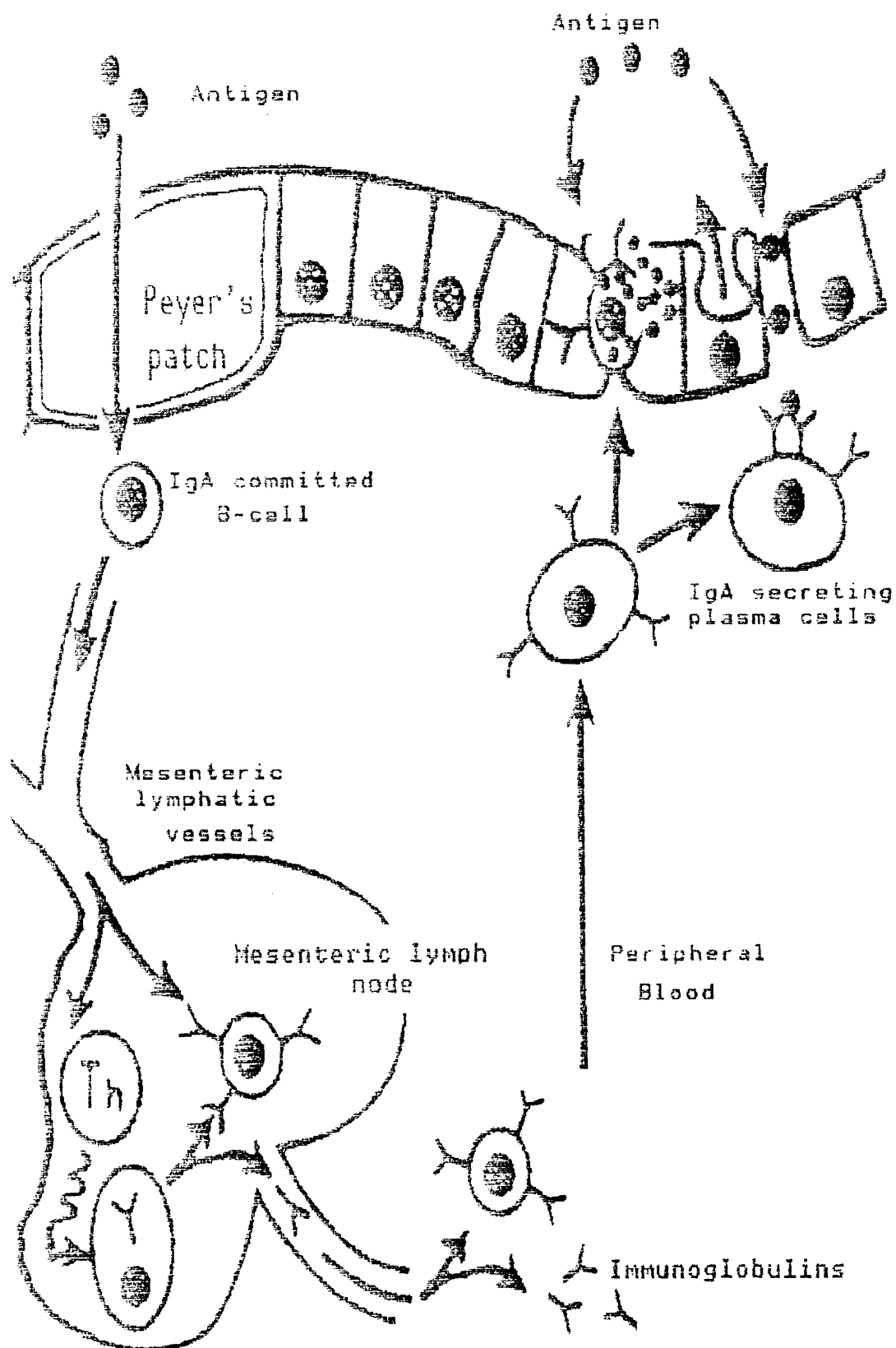
1.1.2. Mucosa associated lymphoid tissue.

The mucosa associated lymphoid tissues (MALT) deal with a large antigenic load comprising principally of inhaled and ingested antigen from the respiratory and gastrointestinal tracts.

The most highly organized MALT occurs in the gastrointestinal tract, referred to as GALT (gastrointestinal associated lymphoid tissue), and has been estimated to contain the largest accumulation of lymphoid tissues in the human body (Brandtzaeg 1985). GALT is of importance as it is responsible for the processing of a large number of food antigens and for the immune response to a variety of pathogenic and commensal organisms. The functions of GALT are diverse as, on the one hand the integrity of the mucosa must be maintained against possibly pathogenic micro-organisms, whilst on the other, food antigens must be recognized and handled without inducing harmful hypersensitive reactions (Kilian et al., 1988).

The components of GALT may be divided into two major parts. First are the organized tissues which comprise the Peyer's patches (PP), isolated lymphoid follicles and the mesenteric lymph nodes (MLN) (Fig 1). Second the lymphoid

Figure 1. Schematic diagram of the gastrointestinal associated lymphoid tissue.



cells which exist in the walls of the intestine both in the lamina propria (LP) and the epithelial layer and are termed intra-epithelial lymphocytes (IEL). These two groups are inter-related to form a system.

1.1.3. Peyer's Patches.

Peyer's patches are collections of lymphoid follicles distributed throughout the small intestine from the pylorus to the ileocaecal valve in a wide variety of animals including birds, rodents and man (Kagnoff, 1981; Owen and Bhalla, 1983).

Their structure was first described by Johann Peyer in 1677. There are usually 20-30 PP in the human intestine and 12-14 in the rat intestine. They are oval, dome-like structures in shape, 1-4 cm long in humans and 0.5-1 cm in rats. PP are located in the mucosa and submucosa with each patch having an extensive blood supply (Bhalla et al., 1981). PP have a cellular structure comparable to that of lymph nodes, containing parafollicular and follicular areas surrounding germinal centres (Parrott, 1976). Within the PP approximately 60-70% of cells are pre-B cells and B cells. These are situated in the follicles with relatively few mature plasma cells (Lyscom and Brueton, 1982; McWilliams et al., 1974). Germinal centres within PP are sites of antigen-induced, T cell dependent proliferation. They are also the areas in which pre-B and B cells differentiate into plasma cell precursors (Carlson and Owen, 1987). In PP germinal centres the majority of B-cells are surface

IgA positive with few carrying IgG or IgM. Surface IgA is not characteristic of germinal centres of all lymphoid tissue but is limited to the secretory immune system and MALT (Kraal et al., 1982).

T cells, predominantly of the T-helper subset, populate the parafollicular areas and constitute the remaining 25-35% of cells in PP. Various accessory cells involved in antigen presentation (eg dendritic cells) are also found in small numbers (Lause and Bockman, 1981).

PP are involved in the primary stages of a local immune response as the site at which luminal antigens are sampled and where naive immature antigen-specific lymphocytes first become activated. Macromolecular and even particulate uptake across the intestine is the first stage in a mucosal response (Volkheimer and Schulz, 1968; Schreiber, 1974; Cook and Olsoa, 1979).

PP are covered on their luminal surface by specialized epithelial cells termed M cells which are involved in the uptake of luminal antigens by a process of endocytosis (Bockman and Cooper, 1973). M cells lack fully developed microvilli, having surfaces that consist of microfolds in humans, and possess a thin apical cytoplasm (Owen and Jones, 1974). Numerous vesicles beneath the apical membrane are formed by endocytosis, and transport macromolecules and microorganisms through the M cell and into the PP (Owen, 1977; Wolf et al., 1981). In one set of experiments mice were given drinking water containing latex particles with a mean diameter of 2 μ m. Latex particles were first recovered from the PP

then subsequently the villi and mesenteric lymph nodes (LeFevre et al., 1979). Further, in mice given oral infections with Salmonella typhi the PP were the first sites of appearance of the organism (Carter and Collins, 1974). Although the main role is transport of luminal antigens it is still unclear if M cells play any part in antigen presentation.

1.1.4. Mesenteric Lymph Nodes.

Once activated by luminal antigens in the PP lymphocytes migrate to the mesenteric lymph nodes (MLN) (Reynolds and Pabst, 1984). MLN drain the mesenteric lymphatics which originate in the intestinal mucosa and PP. Their structure is identical to peripheral lymph nodes with well-developed T cell dependent areas (TDA), primary follicles and germinal centres. Efferent lymph from the MLN drains into the thoracic duct, via the cysterna chyli in the rat, and is the major constituent of the lymph returning to the bloodstream via this route (Mowat, 1987).

MLN contain predominantly T cells but show a majority of the helper subset unlike other systemic peripheral lymph nodes (Durkin et al., 1981). T cells with receptors for IgA (Kiyono et al., 1982) are relatively common and many MLN B cells carry surface IgA (Stevens et al., 1982). MLN contain many mucosa-homing lymphoblasts and lymphocytes originally primed in the PP (Pugh et al., 1983). These remain in the MLN for a period before continuing on to the intestinal mucosa or other

mucosal sites. MLN are also the source of IgA-producing B cells bound for the gut mucosa, with almost all immunoglobulin-producing cells within MLN of the IgA isotype (Cebra et al., 1977).

MLN contain T helper cells capable of inducing terminal differentiation of IgA-committed B cells (Kawanishi et al., 1983). Prior commitment to IgA synthesis in B cells is caused by isotype specific T cells in PP.

To summarize, a local immune response is instigated by luminal antigen being taken up by the M-cells of the PP. The antigen stimulates T and B cells within the PP. These cells once activated by antigen migrate to the MLN where maturation occurs producing antibody-secreting plasma cells and T cell proliferation. From the MLN cells migrate back to the mucosal lamina propria or perhaps to distant mucosal sites providing a specific immunity common to all mucosal surfaces. Such distant expression of immunity following immunization at a different site is the basis of the concept of a common mucosal immune system (See Section 1.2).

1.2 IMMUNOGLOBULINS IN MUCOSAL SECRETIONS.

IgA is the major immunoglobulin isotype in external secretions (Lam, 1976; Tomasi and Grey, 1972). The majority of IgA in secretions is produced locally by plasma cells in the mucosa and sub-mucosa (Kagnoff et al., 1972, 1973). In GALT the plasma cells for local

immunoglobulin production reside in the lamina propria and consist of B-cells previously primed by gut antigens in PP (Kagnoff, 1977). Although IgA is the predominant mucosal immunoglobulin antibody, responses at mucosal surfaces are not restricted to IgA. In GALT both IgG and IgE are important during certain parasitic infestations (particularly IgE) and in association with intestinal disease states (eg IgG in inflammatory bowel disease and coeliac disease).

Lack of IgA in serum and secretions is one of the most common immunological deficiencies (Frommel et al., 1973; Kolstinen, 1975) but unexpectedly gastro-intestinal infections are not a feature of IgA-deficient individuals (McGhee and Michalek, 1981). Possible explanations are the quantitative replacement of IgA-cells in the intestinal mucosa by IgM-cells. Transport of polymeric IgA into external secretions also allows for the transport of polymeric IgM (See Section 1.2.4). In normal individuals transport of IgM into secretions is negligible compared with IgA but some in IgA-deficient individuals IgM appears to be acting as the surrogate choice for secretory IgA. Results from measurements of IgM in the secretions of these individuals show appreciable amounts of IgM far greater than would normally be found. The IgM is associated with secretory component just as IgA would be in normal individuals (McGhee and Michalek, 1981).

Selective IgA deficiency has been associated with intestinal diseases such as coeliac disease. Furthermore

these individuals have an increased incidence of autoimmune and connective tissue diseases, autoantibodies in the absence of overt disease, allergic disorders, and malignancy (Ammann and Hong, 1980). They also have increased levels of antibody to food antigens and circulating immune complexes (Cunningham-Rundles et al., 1978).

In normal human intestinal lamina propria there are relatively few IgG producing cells and IgG in secretions is derived mainly from serum by transudation with little local production (Kagnoff, 1987). In some disease states such as coeliac disease IgG is found in increased amounts in secretions and the number of IgG-producing cells is increased (Kagnoff, 1987). It is worth noting that IgG is capable of fixing complement by the classical pathway and can participate in target cell damage by directing the specificity of antibody-dependent complement-mediated cytotoxic reactions (Kagnoff, 1987), unlike IgA which has been shown to be ineffective in fixing complement (Russel-Jones, 1980). In this way it may be responsible for some tissue damage.

1.2.1. Structure of IgA.

Beta myeloma proteins were shown by Slater et al. (1955) to be antigenically distinct from normal "gamma-globulin" and he postulated the existence of an unknown beta-globulin in human serum. Heremans et al. (1959) found that the precipitate from a conventional zinc sulphate test (Kunkel, 1947) on normal serum contained

part of the "gamma-globulin" but none of the "beta2-globulin" as seen under immunoelectrophoresis. This observation led them to develop a fractionation procedure to purify the "beta2-globulin" which led to the first isolation of IgA from serum. This was followed in 1961 by Tomasi who discovered evidence of this protein in external secretions. The preparation, termed "beta2a-globulin", was shown to possess antibody activity (Heremans et al., 1963) and the same four chain structure as "gamma-globulin". The protein was finally renamed IgA by international agreement (Ceppellini et al., 1964).

Once isolated, Hanson (1961) discovered that IgA predominated in milk. It showed distinctive and unique characteristics which were demonstrated to be due to an additional polypeptide, secretory component, by Tomasi et al. (1965). S-IgA was found to be secreted by local plasma cells in the mucosa and sub-mucosa (Tomasi and Bienenstock, 1968) as a dimer containing an additional polypeptide, J-chain (Koshland, 1975) (Further discussion of J-chain and SC can be found in Sections 1.2.2 and 1.2.3 respectively).

In humans serum IgA was found to be monomeric in form (Tomasi and Bienenstock, 1968). Curiously though when other species were examined it was found that the majority normally had dimeric serum IgA (Vaerman, 1973).

Both molecular forms of IgA were based on the monomer with the typical four chain immunoglobulin structure consisting of two alpha heavy chains and two light (L) chains (Mestecky, 1976). IgA shared the ability

to form polymeric forms with IgM, both IgA and IgM heavy chains possessing a special C-terminal extension which contained an extra cysteine not found in other immunoglobulin heavy chains (Underdown and Schiff, 1986). This extension has been shown to participate in cross-linking monomer sub-units together with inter-chain disulphide bridges (Prahl et al., 1971). Disulphide-bridges were formed between both monomers and J-chain which formed an indirect link between the two monomers (Garcia-Pardoe et al., 1981). SC has also been identified as being covalently linked to the IgA monomers in S-IgA but the site of attachment is as yet undefined (Underdown et al., 1977; Underdown and Switzer, in press).

1.2.2. J-chain - Structure and function.

J-chain was first identified in 1966 by two groups (Rejnek et al., 1966; Cederblad et al., 1966). J-chain was seen as an electrophoretically fast-migrating polypeptide under electrophoresis in alkaline urea-containing sieving gels with L-chain preparations from S-sulphonated or reduced-alkylated human colostral IgA.

At first there was some confusion as to whether this polypeptide was in fact secretory component (SC) (Hanson and Johansson, 1967), but Halpern and Koshland (1970) identified them as distinct proteins. They found J-chain in both human and rabbit secretory IgA and proposed the name "J-chain" because of its possible role in the formation of IgA polymers. A similar polypeptide was found in macroglobulinaemia IgM proteins (Kownatzki,

1971) and was found to be indistinguishable from the J-chain of Halpern and Koshland.

J-chain was characterized as a small glucosamine-containing glycoprotein which is extremely acidic in character due to a high glutamic and aspartic acid content (Mestecky et al., 1972). Halpern and Koshland (1970) suggested a role for J-chain (Joining chain) in the polymerization of IgA. The discovery of J-chain in another polymeric immunoglobulin (IgM) strengthened this argument, as did the lack of J-chain in other monomeric immunoglobulin isotypes (IgG, IgD and IgE) (Koshland, 1975). The reason for this difference between isotypes was made clear by Prahl et al. (1971) who showed that J-chain was linked by disulphide bridges to cysteine residues in the C-termini of IgA and IgM immunoglobulin heavy chains. This site was not present in IgG, IgE or IgD immunoglobulin heavy chains (Garcia-Pardoe et al., 1981).

J-chain is only normally found in the extracellular environment associated with polymeric IgA and IgM (Koshland, 1984). Extracellular free J-chain is uncommon due to its susceptibility to digestion by proteolytic enzymes. However immunofluorescence studies have shown J-chain intracellularly within plasma cells expressing isotypes other than IgM or IgA. What role if any J-chain is performing within these cells is still to be determined.

Studies of human and mouse J-chains have shown considerable homology (77%) in their primary structure

together with similar molecular weight and amino-acid composition (Mole et al., 1977; Cann et al., 1982; Max and Korsmeyer, 1985). This would indicate that J-chain is a highly conserved molecule.

Sequence homology between J-chain and immunoglobulin chains is poor but recent computer assisted sequence analysis data and circular dichromism measurements of denatured and renatured human J-chain suggest structural similarities to immunoglobulin domains (Zikan et al., 1985). These data suggest that J-chain displays beta-sheet folding as occurs in immunoglobulin chains. It is therefore possible that J-chain may possess an immunoglobulin-like fold which could interact with the C4 or C3 regions of IgA or IgM forming domain associations which could aid in stabilizing the polymeric structure (Mestecky and McGhee, 1988).

The number of J-chains per polymeric IgA molecule remains unresolved. Although J-chain was first assumed to be found at a valency of one chain per polymeric IgA molecule there have been several polymeric IgA myeloma proteins isolated which appear to have less than one J-chain per polymer or no J-chain at all (Brandtzaeg, 1976; Tomasi and Czerwinski, 1976). Some myeloma proteins isolated possess more than one J-chain per dimeric IgA molecule (Ricardo and Inman, 1974; Grubb, 1978). However accurate determination of J-chain content within polymeric IgA is complicated by the susceptibility of J-chain to digestion by proteolytic enzymes (Koshland, 1985).

The question as to whether J-chain is required for the polymerization of two IgA monomers or whether it is merely associated with one of the subunits is still uncertain. Cellular analysis of IgA production using pulse-chase experiments has shown that the addition of J-chain is at a very late stage and would appear to occur after polymerization (Koshland, 1985). It may be that J-chain is not directly responsible for IgA polymerization but rather acts to stabilize the structure once formed. If this is so then further work will need to be done to identify more precisely the major role if any of J-chain.

1.2.3. Secretory component - Localization and structure.

Secretory IgA was isolated in 1961 by Hanson and shown to have a greater molecular weight than serum polymeric IgA. These results led researchers to speculate that S-IgA contained additional proteins. To determine if other proteins were involved Hanson (1961) produced antiserum to S-IgA isolated from human milk. Absorption of this antiserum to S-IgA with human serum IgA was unable to eliminate the specificity for S-IgA. Tomasi et al. (1965) used further controls in which antisera to serum IgA were absorbed with S-IgA. This resulted in all detectable reactivity for serum IgA being removed. These results demonstrated antigenic differences between serum and secretory IgA and led to the subsequent isolation of SC using dissociation experiments and immunoprecipitation techniques (Hanson and Johansson, 1967; Bull and Tomasi, 1968).

The molecular weight of SC has been determined as approximately 50 kD - 90 kD depending on the methods used for preparation (Kobayashi, 1971; Heremans, 1974; Brandtzaeg, 1985). Eiffert et al. (1984) have conducted an analysis of human free SC isolated from intestinal secretions and have shown that it consists of a single polypeptide chain with 553 amino acids. The molecule contains 20% carbohydrate attached by N-glycosidic bonds to asparagine residues as 5-7 oligosaccharide side chains. Similar studies using rabbit polyimmunoglobulin receptor (corresponding to the human membrane form of SC) (Mostov et al., 1984) have demonstrated remarkable similarities in their general structure with a sequence homology of 60%.

SC has been found in three separate situations;

1) Epithelial cells and hepatocytes express SC as a surface membrane protein where it is involved in IgA transport, functioning as a receptor for polymeric immunoglobulins (Mostov et al., 1982). In this situation SC has an additional transmembrane tail region of approximately 60-70 amino acids when compared with free SC obtained from external secretions. The tail region is lost once SC is released from the apical surface of the cell whether complexed with polymeric immunoglobulin or not.

2) Subsequently it is also found as a component chain of S-IgA and S-IgM and as a free glycoprotein in external secretions (Weiker et al., 1975; Altamirano et al., 1980).

3) The presence of free SC in external secretions has been demonstrated under normal conditions for all species studied. This is of importance as it implies that production of SC by epithelial cells is a constitutive function which is non-inducible. This results in a constant production of SC by epithelial cells independent of the levels of IgA in the interstitium of the mucosa. SC produced in excess is transported and released as free SC into external secretions. SC has been found using immunofluorescence both free and associated with IgA in the external secretions of all normal individuals and in some IgA-deficient patients where it is associated with IgM (Thompson, 1970; Arnold et al., 1978).

The discovery of SC associated with S-IgA led to the search for the cellular sites of SC synthesis. Immunofluorescence studies of mucosal tissue sections revealed no staining for SC within plasma cells producing IgA (Tomasi et al., 1965). SC appeared to be localized in epithelial cells with later work showing that SC is produced by epithelial cells of both secretory glandular and mucosal surfaces (Heremans, 1974; Tourville et al., 1969). The results led to the proposal of a model for the individual production of IgA and SC by plasma and epithelial cells and a possible mechanism by which these components would combine to produce the S-IgA molecule. This model included the transport of IgA into external secretions and will be dealt with in the next section.

Experiments with free SC in vitro have shown that it binds specifically polymeric IgA and IgM (Mach, 1970;

Weiker and Underdown, 1975; Kuhn and Kraehenbuhl, 1979). SC-binding stabilizes the quaternary structures of both IgA and IgM as shown by the reduction in their dissociation under a range of reducing conditions (Brandtzaeg, 1977, 1985). This stabilization would aid in preventing the breakdown of the immunoglobulins in the gastro-intestinal tract under strong reducing conditions.

SC has a high carbohydrate content which is thought to be responsible in part for increasing the hydrophilicity of S-IgA and IgM (Mestecky et al., 1974; Tomano et al., 1978). Again this would be of value at the mucosal surface increasing the hydrophilicity of antibody-bound micro-organisms so reducing their ability to adhere to the hydrophobic surface of epithelial cells.

There is also evidence that SC-binding increases resistance to a variety of proteolytic enzymes commonly found in the gastrointestinal tract (Lindh, 1975). This would increase the half-life of S-IgA in the lumen and so extend the degree of humoral immunity at the mucosal surface.

1.2.4. Transport of IgA to the mucosal surface.

Transport of IgA to the mucosal surface is essential in order for it to perform the primary function of antigen exclusion (For a further discussion of IgA effector functions see Section 1.2.5). In several early studies it was speculated that SC might facilitate the entry of extracellular IgA into secretory epithelial cells which would then release it into the lumen. This

suggestion was prompted by the demonstration that secretory IgA bore SC as a regular subunit but there was no obvious explanation for its postulated transport function. SC had been shown as a membrane bound protein of epithelial cells on mucosal surfaces (Heremans, 1974; Brandtzaeg, 1985) and was identified as a receptor for polymeric immunoglobulin using in vitro assays and immunofluorescence techniques where anti-SC sera blocked IgA binding (Crago et al., 1978). Further work demonstrated that digestion of surface proteins from living epithelial cells inhibited polymeric IgA binding. Subsequent reexpression of SC on the membranes restored their ability to bind polymeric IgA (Nagura et al., 1979). S-IgA, already associated with SC, was incapable of binding to epithelial cells or of being transported from the interstitium.

Several models were proposed for the actual mechanism involved and in 1973 a common glandular transport model was proposed for dimeric IgA and pentameric IgM which is still accepted. In this model it was suggested that plasma membrane incorporated SC initially bound polymeric immunoglobulin by noncovalent interaction subsequently forming disulphide bonds (Weiker et al., 1975). The complex would then undergo endocytosis (Jones et al., 1982) with SC carrying IgA across the cell within membrane bound vesicles to the luminal face where it would be released as a single unit (S-IgA) by proteolytic cleavage of the membrane portion of the SC (Renston et al., 1980). Binding of polymeric

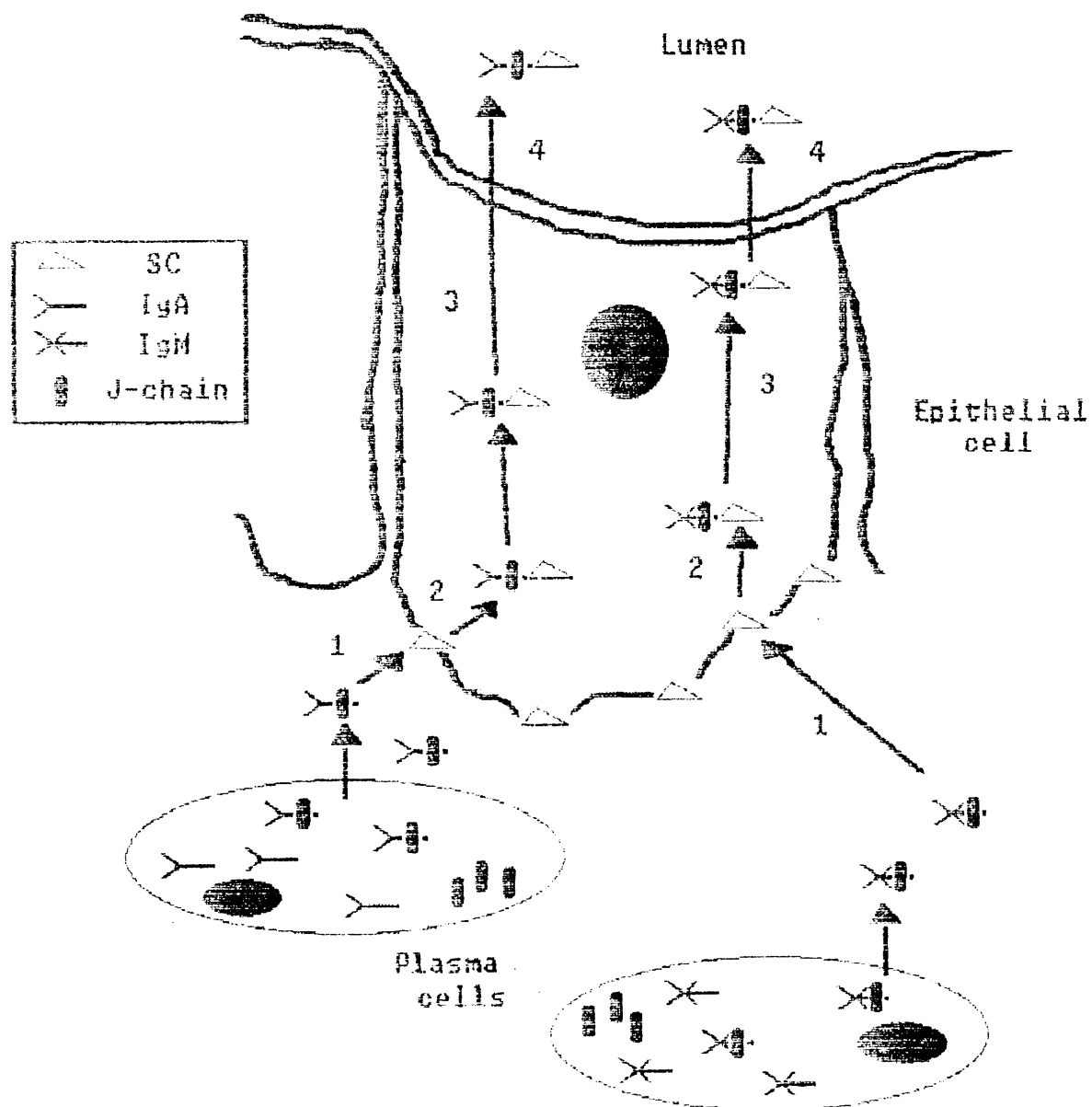
immunoglobulins by SC had been shown and further in vitro studies estimated the affinity of SC for all polymeric immunoglobulins at greater than 10^{10} M^{-1} , sufficient to be considered irreversible (Schiff et al., 1979).

Evidence for the cellular transport by SC was established in vivo by immunofluorescent labelling of tissue sections for IgA and SC (Poger and Lamm, 1974; Brandtzaeg, 1974). Also various in vitro models using colonic carcinoma cell lines (Nagura et al., 1979) and normal dissociated intestinal epithelial cells (Brandtzaeg, 1978) were used to follow transport by immunolabelling of IgA and SC and electron microscopy.

In summary (see Fig 2) IgA transport is now known to be mediated by SC acting as a "shuttle" (1) binding polymeric immunoglobulin at the basolateral surface of epithelial cells then (2) transporting it intracellularly (3) to the luminal surface where it is (4) released into the lumen.

The loss after transport of SC with IgA is unique in intracellular transport systems and was at first used as an argument against the role of SC as a transport molecule. However the very high binding affinity for polymeric immunoglobulins means that loss of SC is perhaps inevitable even though it results in a system which requires the continual renewal of the transport molecule itself. Further studies need to be carried out to ascertain the fate of the membrane bound region of SC after cleavage at the luminal

Figure 2. Secretory component (SC) mediated polymeric immunoglobulin transport.



face of the epithelial cell and to identify the protease responsible for the cleavage of the plasma membrane incorporated region of the SC-IgA complex which is as yet unknown. It has been suggested that the protease may be non-specific as many of the enzymes of the gastrointestinal tract are capable of cleavage of SC-IgA complexes from epithelial cell membranes under in vitro conditions (Underdown et al., 1986).

The preferential transport of polymeric IgA by SC as compared with monomeric IgA has led to the suggestion that J-chain is involved in the binding of SC and polymeric IgA as it is the only peptide both common and exclusive to polymeric IgA and IgM (Brandtzaeg, 1985). Results of experiments using IgA polymers isolated from patients with IgA gammopathies which vary in their heavy chain composition but have their full complement of J-chain reveal that many isolates vary in their ability to bind SC (Schiff et al., 1985). These results would imply that the heavy chain of IgA is the important factor in the binding of SC. Further evidence comes from experiments where the exposed regions of J-chain within IgM were digested by subtilin without destroying SC binding ability (Underdown et al., 1986).

The binding of polymeric IgA by SC has also been shown to be influenced by molecular weight, such that polymeric IgA-antigen complexes are transported less rapidly than polymeric IgA alone (Schiff et al., 1983).

Secretory component (SC)-mediated transport of polymeric IgA has now been shown to occur in most mammals

including man (Brandtzaeg, 1981, 1985). Various mucosal sites around the body such as the gastro-intestinal tract, respiratory tract, ocular tissue, salivary glands, urinary tract, uterus and skin have all shown SC-mediated IgA transport (Jonard et al., 1984; Sullivan et al., 1984; South et al., 1966; Wira et al., 1985; Underdown et al., 1986).

Hepatocytes of certain mammalian species, especially the rat, also produce SC (Vaerman et al., 1982) and in these species blood to bile transport of IgA by SC-bearing hepatocytes has been demonstrated as an alternative route whereby systemic polymeric IgA may reach the gastro-intestinal tract (Jackson et al., 1978; Orlans et al., 1978). The importance of this route in these animals is unclear but it has been suggested that there may be a lack of efficient local transport at the mucosal surface leading to a transudation of polymeric IgA into the systemic circulation. The hepatobiliary transport would act as a "scavenging" system redirecting the polymeric IgA into the gastro-intestinal tract.

Initial work which indicated raised levels of IgA in patients suffering liver dysfunction suggested that the hepatobiliary route might also be of relevance in man (Kutteh et al., 1982; Newkirk et al., 1983). However recent work suggests that there is little or no transport of IgA by the hepatobiliary shunt in man with the raised levels of polymeric IgA in liver disease being due to a lack of breakdown of IgA (Schiff et al., 1985; Delacroix et al., 1982).

Another suggested role for the hepatobiliary route is the immune elimination of low molecular weight antigens in the form of immune complexes with polymeric IgA (Russel et al., 1983). This has now been largely discounted due to the molecular weight sieving effect which reduces the size of antigens capable of clearance by this route (Socken et al., 1981). The successful immune clearance of antigens of all sizes is of importance for all species and yet hepatobiliary transport appears efficient in only some (ie rat, chicken and rabbit). The clearance of small molecular weight antigens is now viewed as a fortunate by product.

1.2.5. Effector roles of IgA.

Effector roles for IgA in secretions have been looked at in greater detail than those in serum (Underdown and Schiff, 1986). The serum levels of IgA mature slowly over approximately 15 years in humans, whilst secretory IgA in external secretions is capable of reaching adult levels in as little as 4 to 6 weeks from birth (Gleeson et al., 1982). Results of this nature have lent weight to the view that IgA is only of importance in the external secretions and to minimize the possible role which it might play in the systemic circulation.

Although IgA is present at a concentration of 2-4 mg/ml in human serum, compared with IgG at 8-16 mg/ml, (Mcconnel et al, 1982) it's function is still as yet poorly understood. Serum IgA is produced mainly by the bone marrow and is exclusively monomeric in form

(Delacroix, 1985). The level of production is comparable with that of IgG in humans and lower serum levels are due to a shorter half-life. Serum IgA is catabolised by the liver at a higher rate than IgG (Heremans, 1974).

Serum IgA has been described as a poor opsonin in conventional assay systems using peripheral blood as a source of phagocytic cells (Wilson, 1972; Wilton, 1978). IgA does not appear to activate complement via the classical pathway (Heremans, 1974). Activation via the alternative pathway has been demonstrated in vitro (Pfaffenbach et al., 1982) but with far less efficiency than both IgG and IgM (Rits et al., 1987). Activation in vivo is generally thought to be relatively ineffective (Griffiss, 1983).

From these results it has been difficult to resolve an effector role for serum IgA. However a hypothesis has been advanced for a role based on its lack of effector functions in serum by Griffiss. He proposed that serum IgA competes for antigen with other isotypes and so acts as a "blocking" antibody (Griffiss et al, 1982). By "blocking" the binding of IgG and IgM and interfering with complement fixation by these isotypes serum IgA would protect the antigen or pathogen from phagocytosis and the whole range of complement mediated antibody dependant host defense mechanisms (Griffiss and Goroff, 1983; Russel-Jones et al., 1980). Under normal conditions serum IgA would act as a "regulating" isotype keeping an equilibrium between hypo and hyper systemic immune responses (Griffiss, 1982b).

Griffiss proposed a specific disease model of this serum IgA regulation and its possible deleterious consequences in meningitis where overproduction of IgA against Neisseria meningitidis could result in the clearance of bacteria by IgG and IgM via complement mediated mechanisms being "blocked" so leading to a systemic bacteraemia (Griffiss, 1982a; Griffiss, 1983). Clinical findings of raised levels of serum IgA specific for N. meningitidis in groups of patients suffering meningitis during epidemics have been quoted as evidence supporting this hypothesis (Griffiss, 1975).

The role of serum IgA is as yet still unclear but the investigation of the role of secretory IgA has yielded more positive results. It is at the mucosal surface that IgA has the greatest effects on immune responses and the three areas of its action (immune exclusion, elimination and modulation) will be covered below in more detail. In the final section the comparison of serum IgA with other serum isotypes is examined as related to opsonic capability, an important requirement in protection against invasive pathogens. The assessment of the opsonic potential of antibody by a luminol-dependant chemiluminescence assay system is examined as a method of comparison used in the present study.

1.2.6. Immune Exclusion.

Secretory IgA is thought to act at the mucosal surface principally by agglutination. The ability of polymeric IgA to cross-link antigens is in itself

beneficial as aggregation of microorganisms in the mucosal lumen would retard their movement to the mucosal surface and impair invasion of the host epithelial cells. The polymeric nature of IgA would also mean increased avidity for antigen.

Secretory IgA has also been shown to prevent adherence of pathogens and toxins by altering their physico-chemical properties so leading to clearance by mucus and peristalsis. Inhibition of the adherence of microorganisms to host epithelial cells by S-IgA has been shown by many investigators (Abraham and Beachey, 1985; Liljemark et al., 1979; Reinholdt and Kilian, 1987). Adherence of microorganisms to host cells may involve both non-specific and specific factors such as hydrophobic interaction with the host cell surface and the use of adhesins to host cell surface receptors (Abraham and Beachey, 1985). The binding of antigen-specific S-IgA antibodies to naturally hydrophobic Escherichia coli and Salmonella typhimurium and reduces their hydrophobicity (Magnusson et al., 1979). This effect is not shared by IgG or serum IgA (Edebo et al., 1985).

Attachment of viruses and bacteria at the mucosal surface is often associated with the presence of microbial adhesins and of specific receptors on target cells. IgA, by binding to the organism or toxin could interfere with this attachment either by direct blocking, steric hindrance or a conformational change in the receptor binding molecule on the organism. Purified

secretory IgA antibodies directed against specific structures such as fimbriae on gonococci and enterobacteria have been shown to prevent attachment of bacteria to mucosal surfaces (Svanborg-Eden et al., 1983; Tramont et al., 1980).

Secretory IgA binding could render a pathogen susceptible to other non-specific anti-microbial factors such as mucins, lysozyme and peroxidase by reducing its mobility and preventing adherence. There is also evidence that S-IgA can potentiate the effects of some non-specific anti-bacterial factors. Arnold et al. (1984) showed that IgA antibodies specific for iron-binding proteins on bacterial surfaces exerted a powerful bacteriostatic effect which synergized with iron-unsaturated lactoferrin. This effect was seen with many enteric bacteria including E. coli and

Pseudomonas aeruginosa. The mechanism involved is unclear but may be related to the findings that some of the lactoferrin in external secretions is covalently bound to S-IgA (Watanabe et al., 1984).

Even if the pathogen succeeds in binding to its target cell, binding by S-IgA could still have a beneficial effect. Taylor et al. (1985) have shown that influenza virus coated with neutralizing IgG is still capable of invading its target cell in an in vitro cell assay. Using specific secretory IgA the virus was shown to still attach but could no longer be internalized. Further work will be needed to elucidate the mechanisms involved.

IgA also possesses a role as anti-toxin in many entero-toxigenic infections including cholera. Toxin neutralization by S-IgA has been shown to occur by sterically blocking their binding to target cells or substrates much the same as with other systemic isotypes. However toxin neutralization within the gastro-intestinal tract is particularly relevant due to the large number of diseases of the gut caused by entero-toxin producing bacteria (Holmgren et al., 1972; Brown, 1986).

1.2.7. Immune Elimination.

Immune clearance from systemic circulation of polymeric IgA-antigen complexes has been shown to occur via SC-mediated transport provided the antigen is of a low molecular weight. IgA transport from mucosal sites whilst complexed with low molecular weight antigens has been proposed as a method for the removal of low levels of dietary antigens which cross the gastro-intestinal epithelium. Without clearance systemic responses could result in allergic reactions.

Elimination of antigen from systemic circulation by systemic antibody primarily occurs by opsonization and phagocytosis. Initial studies of the opsonic abilities of IgA produced poor results (Wilson, 1972; Wilton, 1978). However these early experiments used phagocytes obtained from peripheral blood. Studies since have shown a marked bias in Fc-receptors for IgG as opposed to IgA in macrophages and polymorphonuclear leucocytes from peripheral blood (80% and 20% respectively) (Fanger et

al., 1980). Phagocytes from mucosal sites such as alveolar macrophages show increased opsonization when using IgA as an opsonin when compared with using IgA as an opsonin with peripheral blood macrophage controls (Richards and Gauldie, 1987). This is reflected by the greater number of Fc-receptors for IgA on alveolar macrophages both in number of cells and number per cell (Fanger et al., 1983a). From these results it would appear that IgA can aid antigen elimination via opsonic phagocytosis by cells at mucosal sites. The localization of Fc-receptors to cells from mucosal sites is also explained by the findings of Fanger et al (1983b) who demonstrated that incubation of alveolar macrophages with IgA for 24 hours prior to assay led to a greatly increased number of cells bearing Fc-receptors for IgA, suggesting induction of receptors by the presence of IgA.

1.2.8. Immune modulation.

Immune modulation enables the immune system to regulate responses which might be destructive if expressed systemically. IgA has been proposed to perform immune modulation by a damping down of systemic responses by competing with IgM and IgG for antigen at mucosal sites as discussed above (Griffiss, 1983).

Secretory IgA also appears to regulate immune responses at the mucosal surface. Experiments have shown that antigen complexed with IgG or IgE result in an increased absorption of various bystander antigens through both oral and gastrointestinal epithelium (Bloch

and Walker, 1981; Lim and Rowley, 1982). Specific S-IgA antibodies would reduce the uptake of these bystander antigens so preventing a possible systemic response to an increased antigenic load at the mucosal surface. Important antigens include both food and environmental antigens which if not prevented from entering the systemic circulation by uptake from bronchial or gastrointestinal epithelium could result in systemic responses producing allergic reactions.

Another form of modulation is produced by the mechanism of oral tolerance (Kagnoff, 1982). Oral tolerance results from ingestion of large doses of protein or repeated ingestion of particulate matter and eventually leads to systemic hyporesponsiveness when challenged systemically. The phenomena was first noted by Dakin over one hundred and fifty years ago (Richman, 1979) that North American Indians ingested poison ivy leaves to prevent dermatitis upon subsequent contact with that plant. This was subsequently termed the Sulzberger-Chase reaction and has now been described for a variety of substances (oxazolone - Glaister, 1973; ovalbumin - Hanson et al., 1979; bovine serum albumin - Thomas and Parrott, 1974).

The role which IgA may play in the induction of oral tolerance is as yet unclear as the precise mechanisms involved are not yet known. Three major mechanisms have been proposed two of which suggest a role for IgA.

The first suggests oral immunization leads to circulating immune complexes forming on subsequent

parenteral challenge so producing systemic unresponsiveness. Andre et al. (1975) demonstrated that following intragastric administration of sheep red blood cells the serum of treated animals contained an antigen-specific factor which suppressed the systemic humoral response and which was thought to be an IgA antigen-antibody immune complex. Similar results were obtained by Kagnoff (1978).

The second idea was devised by Kagnoff following his earlier work and proposes a role for anti-idiotypic IgA produced by oral immunization which would invoke systemic unresponsiveness once the antigen was administered systemically (Kagnoff, 1980). Kagnoff's work was based on the findings that continued feeding of sheep red blood cells (SRBC) to mice resulted in oral tolerance. It was possible to transfer this suppression of systemic responses to naive animals by serum but not by cells, implying a serum factor as the suppressive agent (Kagnoff, 1978). In vitro studies of cellular responses to SRBC were also found to be suppressed by the serum from mice with oral tolerance. The factors appeared to be immunoglobulin in nature as the activity could be removed by absorption of the serum on an anti-mouse immunoglobulin column (Kagnoff, 1980).

Kagnoff initially speculated that the factor could be antigen-antibody immune complexes as absorption with antigen did not appear to remove the suppressive factor although it did reduce the effect (Kagnoff, 1978). However it was finally proposed that the suppressive

effect was caused by the presence of anti-idiotypic antibodies. It was suggested that the oral feeding induced the production of an IgA mucosal response which led to the production of anti-idiotypic IgA antibodies. These in turn would enter the systemic circulation and induce the systemic hyporesponsiveness demonstrated in tolerant mice.

Most current evidence about oral tolerance suggests that the role of IgA may not be as important as originally thought, with a large number of researchers suggesting rather that the induction of T suppressor cells in the GALT is the key effect. Further work will need to be done to elaborate the mechanisms involved.

1.2.9. Comparison of opsonic capability of serum IgA with other serum isotypes.

Opsonic capability against invasive pathogens is important for a protective antibody (Horwitz, 1982). Opsonization involves the binding of antibody to antigen which enables subsequent binding of the antigen-antibody complex to phagocytes either via antibody specific receptors or by alteration of the physico-chemical nature of the antigen (Horwitz, 1982).

Particles or microorganisms with surfaces more hydrophilic than those of phagocytes have been shown to be resistant to phagocytosis due in part to physico-chemical interaction which prevent them from approaching the phagocytic cell surface (van Oss and Gillman. 1972; van Oss et al., 1975). Phagocytosis has been shown to be

a surface phenomenon (van Oss, 1978). Attachment of specific antibody has been shown to alter the surface hydrophobicity of antigens such that they become less resistant to phagocytosis (Steward, 1974).

Receptors on the phagocytic cell surface specific for differing isotypes of immunoglobulin provide a further method of binding the antigen-antibody complex to phagocytes (Horwitz, 1982).

This binding can facilitate phagocytosis resulting in the uptake of the antigen into the phagocyte within a phagosome. This allows the killing of viable pathogens with the minimal of tissue damage as the bactericidal metabolites (eg hydrogen peroxide) are released intracellularly into the phagosome (Elsback and Weiss, 1983).

The opsonizing activity of antibody has been shown to be mainly a function of the IgG isotype, in humans specifically the IgG1 and IgG3 subclasses (Steward, 1974). The opsonic effect of the IgG molecule appears to be specific to the Fc portion. The Fc portion would be responsible not only for isotype specific interaction with Fc receptors on phagocytes but is also highly hydrophobic so increasing the interaction with phagocytic cell surfaces (Steward, 1974; van Oss, 1978).

IgM in the absence of complement does not appear to act as an effective opsonin. Phagocytes do not appear to carry IgM specific receptors and this may in part explain the lack of opsonization. Hydrophobic interaction may

also play a part as IgM is less hydrophobic than IgG (van Oss and Stinson, 1970).

IgA is the least hydrophobic isotype and shows only poor opsonic activity in conventional assays (van Oss and Gillman, 1972; Kilian et al., 1988). The low number of phagocytic cells in serum bearing Fc receptors for IgA may account for the low level of opsonic activity with this isotype (Fanger, 1980).

Much of this work has been carried out with preparations of immunoglobulins from serum. A study of the properties of these isotypes occurring in low concentrations in the serum is difficult. Myeloma proteins have been used for this purpose but these bear a known antibody specificity only exceptionally. Monoclonal antibodies make it possible to obtain large quantities of antibody having both the specificity and isotype required for comparative work.

The comparison of opsonic activities of the various isotypes could be assessed using an assay sensitive to both the surface interaction and subsequent oxidative burst associated with intracellular killing. The luminol-dependent chemiluminescence assay has been shown to be sensitive to both phenomena and has been shown to be an accurate measure of opsonic activity. Investigation of the opsonic activities of these isotypes would aid in the understanding of the role of IgA in serum which is as yet unclear.

Chemiluminescence (CL) occurs when phagocytic cells come into contact with particulate matter or

microorganisms (Allen et al., 1972). Phagocytic cells are capable of releasing bactericidal oxygen species including superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen. These oxygen species produce CL by their transition of high energy electrons to lower energy states, the energy lost being emitted as light. The level of CL can be amplified by the addition of Luminol (5-amino-2,3-dihydro-1,4-phthalazine-dione a cyclic hydrazide). Luminol-dependant chemiluminescence has been used to investigate the functioning of phagocytic cells and the use of antibodies for opsonization in phagocytic assays (Hemmings et al., 1976; Easmon et al., 1980; Stevens and Young, 1977).

Due to the dependence on cell particle contact and cellular oxidative metabolism, phagocytic chemiluminescence can be used as a sensitive measure of opsonic activity (Stjernholm et al., 1973). Correlation with other assays in clinical use such as the nitroblue tetrazolium (NBT) reduction test and the intraleucocytic killing test have shown chemiluminescence as an accurate measure of opsonic activity (Thomas et al., 1988).

1.3 IGA MONOCLONAL ANTIBODIES AND THEIR PRODUCTION.

The functions of IgA can be subdivided into three groups, those performed by secretory IgA (S-IgA), those performed by polymeric IgA (pIgA), unassociated with SC, and finally those performed by monomeric IgA (mIgA). To understand how each function relates to the relevant

molecular form it would be useful to produce an IgA antibody source of defined specificity capable of being expressed in all three forms.

Investigation of the role of IgA has previously relied on antibody purified from serum or mucosal secretions. Various protocols have been subsequently used to prepare the various molecular forms.

Serum is a poor source of large quantities of IgA in man. IgA levels represent less than 3-4% of total protein content in normal human serum (Heremans, 1974). Purification is difficult as monomeric serum IgA closely resembles IgG and IgE both in molecular size and electrophoretic mobility.

Isolation involving immunoabsorption on a column by a lectin (jacalin) has been described for IgA but the lectin is species specific for human IgA and appears to vary in its reactivity between the two sub-classes of human IgA.

Immunochemical methods for IgA rely on anti-IgA sera. Use of these methods necessitate that the anti-sera is monospecific but to isolate S-IgA in this manner is impossible as the isotype specific Fc fragment is covered by the associated SC.

Mucosal secretions provide a source of large quantities of polymeric IgA but are complicated by the ability of S-IgA to become strongly bound to non-specific proteins (Tomasi and Bienenstock, 1968). External secretions also contain proteolytic enzymes and although not vulnerable to attack (Lindh, 1975) S-IgA

still suffers degradation on storage with enzymic contamination (Kobayashi et al., 1973).

Polymeric IgA isolated from S-IgA necessitates the reduction of the disulphide bonds which join IgA and SC. The reduction can cause denaturing of the preparation.

Purification from both serum and secretions provide polyclonal antibody preparations which present problems as the antibody can be of unknown specificity and heterogeneity in its binding characteristics.

These problems have been overcome in the past by the use of polymeric IgA from subjects with IgA myelomas (Parkhouse et al., 1971). This source produces large quantities but no choice in the specificity of the myeloma. Myeloma IgA produces a wide variety of molecular forms from monomeric to pentameric, with increased levels of polymeric forms compared with normal serum (Heremans, 1974). These polymeric forms are unusual in molecular composition with J-chain, some showing greater than one J-chain per polymeric IgA and some showing no J-chain at all (Koshland, 1985).

It is clear from these results that myeloma proteins are not representative of normally produced serum polymeric IgA and as such may be capable of giving artifactual results. Myeloma IgA is also unrepresentative of mucosally derived IgA where the majority of molecular forms are dimeric.

Most of these problems could be overcome by the production of mucosally-derived IgA monoclonal antibodies. These would represent a reproducible

polymeric IgA antibody source, of defined specificity, without the problems associated with bound-SC in preparations from secretions. This would give the opportunity to characterize the properties of polymeric IgA as distinct from those when linked with SC after transport into external secretions.

The use of conventional techniques for MAb production have resulted in a poor yield of IgA MAbs. Explanations of these results have focused on two main areas. Immunization protocols for MAb production normally use systemic routes (Tiffin, 1987). This immunization is biased in favour of producing a systemic humoral response primarily with IgM or IgG. IgA responses to systemic immunization are very poor with almost no stimulation of secretory antibody (Mestecky and McGhee, 1988). The second problem involves the use of the spleen as a source of lymphocytes for MAb production (Tiffin, 1987). The spleen contains very low levels of surface IgA positive lymphocytes and as such the chances of selecting for an IgA hybridoma are small (Komisar et al., 1982).

Within recent years these problems have been overcome by more complex protocols directed toward producing IgA monoclonals derived from stimulated mucosal tissue. There have been three main approaches which have involved the use of immunization via mucosal routes, the use of mucosally derived lymphoid tissue or a combination of both for hybridoma production.

Methods initially relied on the use of mucosal routes of immunization to produce an IgA response. This

approach was demonstrated by Colwell et al (1982). They used gastric intubation of Balb/C mice to produce immunization of the gastro-intestinal lymphoid tissue, followed by the use of spleens from immunized animals to produce MAbs. This method was partially successful producing a greater number of IgA MAbs than before although these were still in the minority. Komisar et al. (1982) used a different approach using conventional immunization techniques. Balb/C mice were immunized intravenously followed by repeated intraperitoneal challenge. Animals were sacrificed and pooled Peyer's patches and mesenteric lymph nodes were collected and used as a source of lymphocytes for fusion. The yield of IgA monoclonal antibodies was better than that found by Colwell et al. (1982) but the numbers were still low. A combination of mucosal immunization and the use of mucosal tissue for a source of lymphocytes has proved to be the most successful method.

Dean et al (1982) used a more complex protocol in rats, and using surgery, which allowed selective immunization and use of gastro-intestinal associated lymphoid tissue as a source of B cells for hybridoma production. This yielded IgA MAbs almost exclusively. These MAbs are also useful as they have been shown to be transported into the bile in rats when injected intravenously, efficiently providing a route for mucosal targeting of antibody (Dean et al., 1983).

The immunization procedure involves surgery. The intestine is exposed and small quantities (10-15 ul) of

antigen solution are injected into the Peyer's patches (PP). These organs are the areas of initial antigenic stimulation in a mucosal immune response (See Section 1.1.3). Subsequent immunizations are either via PP or intraperitoneally.

Fusion involves the use of either of two sources of primed lymphocytes. First, once primed with booster immunizations mesenteric lymph nodes are removed 3-4 days after the last immunization and used for fusion. A second source of primed lymphocytes necessitates the surgical removal of MLN from the rat prior to the immunization procedure. These rats are then left to recover for approximately 6 weeks and reconstruct a mesenteric lymphatic system without MLN's. Following the immunization procedure a canula is passed into the cisterna chyli, the major lymphatic vessel draining the mesenteric lymphatic system into the thoracic duct. Following canulation the rat is held in a restraining Bollman cage which prevents movement and the displacement of the canula. The efferent mesenteric lymph flowing from the GALT to the thoracic duct, rich in IgA-secreting plasma cells, migrating from the Peyer's patches, is collected via the canula and used as a source of lymphocytes for fusion.

This method yields a majority of IgA secreting hybridomas which produce predominantly dimeric IgA.

1.4 SALMONELLA TYPHIMURIUM AS AN IMMUNIZING ANTIGEN.

In order to investigate role of IgA in mucosal immunity using monoclonal antibodies (Mabs) it was necessary to choose an appropriate antigen as a target for immunization and subsequent studies within this project.

The antigen would have to be capable of eliciting a mucosal response and be of relevance in mucosal disease so that any Mabs produced could be used later in possible disease models either in vivo or using cell-culture in vitro assays. Further the antigen should have defined antigenic determinants which could be screened for. Ideally any choice should possess multiple antigens in order to give the greatest chance of selecting monoclonal antibodies (Mabs).

Salmonella typhimurium was chosen as a suitable antigen against which to produce monoclonal antibodies. S.typhimurium is an important mucosal pathogen with many well defined antigenic structures. It is capable of producing systemic infection in mice and is typical of Salmonella as a genus fulfilling all the requirements for production of disease as shown by a number of pathogenic organisms. These requirements include;

1. Gaining entry to the host by survival and multiplication on host membranes (Takeuchi, 1967).
2. Adhering and penetrating host cells (Gianella et al., 1973).

3. Multiplying and surviving in vivo (Hale and Formal, 1981).

4. Resisting host immune responses both cell-mediated and humoral (Fields et al., 1986).

5. Causing tissue damage (Rout et al., 1974).

Salmonellae are well suited for studies of bacterial disease mechanisms and virulence. Several useful animal models have been described using S. typhimurium which closely resemble human disease. Examples include the typhoid model in mice (Collins, 1969), entero-colitis model in rats (Fahey and Cooper, 1970) and the starved, opiated guinea pig model of invasive salmonellosis (LaBrec et al., 1964). The genetic background of Salmonella has been extensively researched providing information on genotype as related to virulence (Davis et al., 1980).

S. typhimurium infection in man is usually localized to the gastro-intestinal tract rarely causing systemic illness. It is an important cause of food-poisoning (Levine et al., 1983). Mice develop a typhoid-like systemic disease when infected with S. typhimurium and are used as a disease model for typhoid in humans (Akeda et al., 1981). Much work has been carried out on the development of bacterial vaccines for oral administration using S. typhimurium in mice (Hoiseth and Stocker, 1981). It is hoped that using this information an oral S. typhi vaccine may be developed for clinical use in man (Levine et al., 1983).

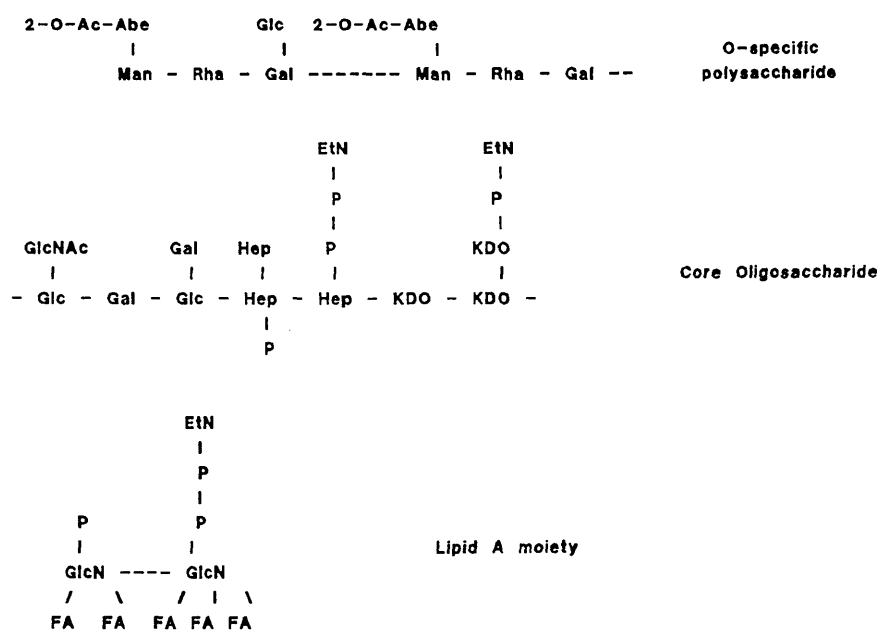
In 1951 Bacon and colleagues identified attenuated auxotrophic mutant strains of S. typhi which required p-aminobenzoate. Based on these findings Hoiseth and Stocker (1981) used transposon (Tn10) mutagenesis to produce similar auxotrophic mutants of S. typhimurium from virulent prototrophic strains. The mutant strains included those which required aromatic compounds such as p-aminobenzoate and various aromatic amino acids such as tyrosine and tryptophan (aro⁻ strains). Aromatic compounds required by these mutants are unavailable in mammalian tissues as mammals do not possess the ability to synthesize them. Due to this aro⁻ strains are prevented from surviving in vivo. Such a strain S.typhimurium (SL3261 BRD026), an aro⁻ A mutant, was chosen as the immunizing strain for the production of IgA monoclonal antibodies. The strain was a construct of an aro⁻ A attenuated strain which carried a plasmid coding for the B sub-unit of the heat-labile (LT) enterotoxin of Escherichia coli (Maskell et al., 1987). The LT-B itself causes no pathological effects. Previous work showed a good mucosal response to the LT-B antigen which paralleled that directed against the Salmonella antigens, with the LT-B being fully expressed (Maskell et al., 1986). LT-B is a highly defined antigen specific for a mucosal environment (Dallas and Falkow, 1980) and it was hoped that some of the selected IgA monoclonal antibodies during the study might be specific for LT-B.

1.4.1. Salmonella typhimurium lipopolysaccharide.

The other main antigen important in the screening of IgA monoclonals would be the endotoxin lipopolysaccharide (LPS). LPS is associated with the effects of septic shock in gram negative bacteraemia and has been implicated as a virulence factor for Salmonella species (Roantree, 1971).

LPS is a structural component of the outer membrane of gram-negative enterobacteria. The polysaccharide portion is essential for the bacteria in resisting the host immune defenses and the lipid portion, or lipid A, is responsible for the toxic activity producing tissue damage (Morrison, 1983). The overall structure of LPS from S. typhimurium is given in Fig 3. Exposed on the surface of the bacterium is the O polysaccharide chain, in which the O antigenic specificity of the bacterium resides (Orskov et al., 1977). The O antigen is the immunodominant molecule of the LPS and has also been shown to be the serologically dominant antigen during infection (Luderitz et al., 1971). The O chain has been shown to be composed of polymerized oligosaccharide repeating units (Robbins and Uchida, 1962). The structure of the repeating oligosaccharide varies between strains belonging to different serogroups. This results in many O-antigens which differ widely between species and within species between different strains (Orskov et al., 1977). Salmonella serology is mainly based on the various O-antigens which vary enormously with over 2000 serotypes identified (WHO, 1980). The O chain is covalently linked to an oligosaccharide chain, the core, which appears to

Figure 3. Structure of Salmonella typhimurium
lipopolysaccharide (LPS).



FA = Fatty acid
EtN = Ethanolamine
GlcNAc = N-acetyl glucose
Glc = Glucose
Abe = Abequose
Hep = Heptose
Rha = Rhamnose
Gal = Galactose
Ac = Acetyl
KDO = 2-keto-3-deoxy-manno-octonate
Man = Mannose

be common to most , or all, Salmonella bacteria (Galanos et al., 1977; Luderitz et al., 1982). The core in turn is covalently linked to the lipid A moiety (Rietschel et al., 1983).

Bacteria which lack the O polysaccharide chain, e.g. rough mutants, have been shown to be normally avirulent (Roantree, 1971) although some exceptions have been isolated (Fields et al., 1986).

Compared to the smooth parent strain the reduction in mouse virulence can be up to a millionfold in initial inocula. The rough mutants can colonize the intestine, are able to adhere to the mucosal surface and can penetrate intracellularly (Tannock et al., 1975). This does not imply that the O antigen is not of importance in virulence. Studies of the adherence of rough mutants with subsequent survival within macrophages has revealed a reduced viability compared to smooth strains which possess the O-antigen (Stendahl and Edebo, 1972). Strains of S. typhimurium isogenic except for their O antigens have been constructed and shown to vary in their virulence dependant on the O antigen expressed (Lyman et al., 1977; Valtonen, 1977). Recent experiments (Mintz and Deibel, 1983) have also shown that smooth LPS may be required for Salmonella invasion of mucosal epithelial cells. LPS has also been implicated as a virulence factor related to host immune resistance. Several studies have implicated LPS (Glynn and Ward, 1970; Munn et al., 1982; Nelson and Roantree, 1967) in resistance of bacterial

strains to the bactericidal activity of serum in both immune and non-immune individuals.

1.5 IN VITRO INVASION MODEL FOR SALMONELLA.

An in vitro invasion model for S. choleraesuis has been described using an epithelial cell line grown on a permeable support (Finlay et al, 1988). The cell line used were Madin-Darby canine kidney cells which are extensively used in experimental work involving transcytosis of molecules across epithelial monolayers (Simmons and Fuller, 1985).

1.5.1. Madin-Darby canine kidney cell-line.

Madin-Darby canine kidney cells were first isolated in 1958 by Madin and Darby (ATCC, 1981). They constitute a polarized epithelial cell-line which when grown on a permeable support form a polarized epithelial monolayer which is impermeable to ions, has a measurable transepithelial electrical resistance and has several defined apical and basolateral surface markers (reviewed by Simmons and Fuller, 1985).

MDCK cells when seeded in tissue culture on a permeable support attach to the support in a defined manner with the basolateral surfaces oriented to the support surface (Misfeldt et al., 1976). Gravity has no effect the basolateral surface always being that side which attaches to the support. The monolayer acts as a

physical barrier between the basolateral and apical medium preventing passage of macromolecules except by transcytosis through the cells of the monolayer. The cells of the monolayer form "tight junctions" between each other which effectively seal the monolayer (Cerijido et al., 1978). These "tight junctions" are comparable to those found between enterocytes in normal gut epithelium.

The monolayer assumes a highly polarized character with a electric potential being generated between apical and basolateral surfaces by the selective transport of sodium ions by apical and basolateral membranes (Cerijido et al., 1978). There is also a polarized uptake of methionine, together with other molecular differences between apical and basolateral membranes including viral receptors (Simmons and Fuller, 1985).

MDCK cell monolayers have been extensively used as an in vitro model of normal epithelium to study the polarity of virus infection and maturation (Rodriguez-Boulan and Sabatini, 1978; Fuller et al., 1984). But the cell line was only first used to investigate bacterial invasion of epithelial cells by Finlay et al (1988).

1.5.2. Invasion of MDCK cells by Salmonella choleraesuis.

S. choleraesuis is a common pathogen of pigs and is also a highly invasive and serious pathogen in humans. Unlike most other non-typhoid Salmonella , S. choleraesuis usually causes invasive disease, often without any gastro-intestinal symptoms. This organism is

considered the prototype of invasive Salmonella (Rubin and Weinstein, 1977).

Recent work with S. choleraesuis has shown that the bacteria can adhere to an MDCK monolayer in a specific manner, attaching preferentially to the apical surface (Finlay et al., 1988). Following adherence to the apical surface the Salmonella invades the cells without apparent cytopathic effects in a manner termed as endocytosis. This endocytosis is apparently mediated by both the bacteria and the MDCK cells. Inhibition of bacterial or MDCK cell metabolism can prevent bacterial uptake by the MDCK cells. Salmonella within the epithelial cells appear to cross to the baso-lateral surface and exit the cells by exocytosis, where they can be collected from the baso-lateral medium. This invasion and subsequent release from the baso-lateral surface of the cells has been termed transcytosis and appears to have little affect on the viability of the MDCK cells or the bacteria. The process is also surface dependant with transcytosis of bacteria from the basolateral to apical surface being minimal.

The course of the transcytosis of S. choleraesuis was followed by electron microscopy studies which show a remarkable similarity to the electron microscopy of S. typhimurium invasion of rabbit ileal loops carried out by Takeuchi (1967). The use of S. typhimurium within this system produces the same results as S. choleraesuis (Finlay, personal communication). Therefore it was hoped that this in vitro model could be used to investigate any

monoclonal antibodies produced against S. typhimurium
during the study.

2 . METHODS AND MATERIALS .

2.1 MATERIALS.

2.1.1. Organisms.

Salmonella typhimurium (SL3261 BRD026) and S. typhimurium (Sl3261) were kindly donated by Dr G. Dougan (Wellcome Biotech, Beckenham, Kent). Neisseria meningitidis (type Y) CN6432 and Salmonella minnesota (wild type strain) CN1091 were obtained from the Wellcome Culture Collection (Wellcome Biotech, Beckenham, Kent).

2.1.2. Cell-lines.

Madin-Darby canine kidney cells (MDCK) were obtained from the Department of Virology, Wellcome Biotech, Beckenham, Kent. Myeloma Y3 Ag1.2.3. was acquired from Dr C. Dean (Institute of Cancer Research, Royal Marsden Hospital, Sutton).

2.1.3. Monoclonal antibodies.

Monoclonal antibodies WMY59 (IgG1), WMY63 (IgM) and WMY67 (IgA) specific for Neisseria meningitidis (type Y) capsular polysaccharide were kindly supplied by Dr R. Lifely (Wellcome Biotech, Beckenham, Kent).

2.1.4. Lipopolysaccharide (LPS) preparations.

Purified lipopolysaccharide (LPS) preparations of S. minnesota (wild type strain), S. virchow (wild type strain), S. minnesota R60 (Ra), R5 (Rc) and R595 (Re)

were kindly donated by Dr N. Mehta (St. Mary's Hospital Medical School, Paddington, London).

2.2 BACTERIAL CULTURE CONDITIONS.

2.2.1. Solid medium.

Neisseria meningitidis (type Y) was serially cultured on GC-agar at 37 °C in an atmosphere of 7% CO₂. Each culture was grown up for 24 hours and harvested by washing the plates in cold phosphate buffered saline (PBS) pH 7.2. The bacterial suspension was washed twice in PBS pH 7.2. The absorbance of the resulting bacterial suspension was measured spectrophotometrically at 540 nm.

Salmonella typhimurium SL3261 BRD026 (SL/BR) and S. typhimurium Sl3261 (SL) were serially cultured on nutrient-agar at 37 °C in an atmosphere of 7% CO₂. Culture were harvested after 24 hours, by washing the plates in cold phosphate buffered saline (PBS) pH 7.2. The bacterial suspension was washed twice in PBS pH 7.2. The absorbance of the resulting bacterial suspension was measured spectrophotometrically at 540 nm.

A viability count was conducted to determine the concentration of colony forming units (CFU) within the bacterial suspension. The bacterial suspension with a known absorbance at 540 nm was diluted in sterile PBS pH 7.2 in a series of 10-fold dilutions. In triplicate, 0.05 mls of each dilution was spread on GC-agar and incubated as above. The number of CFU for each dilution was determined from the mean of three plates. Plates with

greater than 300 colonies or less than 50 colonies were not used. Several bacterial suspensions of differing absorbance (540 nm) values were used.

Subsequent bacterial suspension were routinely adjusted to an absorbance (540 nm) of 0.5. as this was shown to be equivalent to a concentration of 1×10^9 CFU ml⁻¹ suitable for use in the chemiluminescence assays.

2.2.2. Liquid medium.

Salmonella typhimurium SL3261 BRD026 (SL/BR) and S. typhimurium SL3261 (SL) were grown in Nutrient Broth (NB) using one litre baffled Erlenmeyer flasks incubated at 37 C on an orbital shaker (Lab-line Instruments Inc., Illinois, USA) at 250 RPM. Each culture was inoculated with 5 mls from a static flask incubated overnight at 37 C containing either SL/BR or SL. The growth curve of SL/BR and SL for the shaker flask cultures showed that after 4 hours each strain had reached the log-phase of growth.

The cultures were harvested in log phase and washed three times in PBS pH 7.2. The absorbance (540 nm) of the bacterial suspension was measured as above and was then adjusted to the appropriate absorbance value or CFU concentration dependant on whether it was to be used for in vitro adherence assay, physico-chemical analysis or as antigen for radioimmunoassay.

Viability counts were performed as above to determine the CFU concentration as related to absorbance (540 nm).

2.2.3. Storage.

Bacteria were harvested from plates of overnight growth at 37 °C and resuspended in 10% glycerol glucose broth. Aliquots of 1.0 ml were dispensed into cryovials and stored either in liquid phase or gas phase at -120 °C using liquid nitrogen.

2.3 PRODUCTION OF RAT MABS.

2.3.1. Immunization schedule for rats.*

Two groups of two 6 week old, male Hooded rats weighing 200-250 were immunized and resulted in 4 successful fusions using the following schedules;

Group 1 were subjected to mesenteric lymphadenectomy prior to the immunization schedule. These rats were destined for canulation in order to collect efferent mesenteric lymph as a source of primed lymphocytes for fusion.

Once the MLN has been removed, efferent mesenteric lymph (EML) contains primarily lymphocytes predominantly of the IgA isotype migrating from the Peyer's patches. Collection of EML from rats with MLN gives far lower levels of surface IgA positive lymphocytes as the MLN appears to act as a regulatory organ where the immune response is switched to the production of IgG and IgM lymphoblasts. IgA plasma cells from the Peyer's patches migrate to the MLN where they undergo terminal differentiation before the returning to the mucosal lamina propria.

[* For detailed photographs see Section 6. Appendix]

The rat was anaesthetized with diethyl ether and the lower abdomen was opened by making a 2 cm incision along the central line below the diaphragm. The small intestine and caecum were carefully extracted and laid onto a surgical swab soaked in sterile saline. The lymph nodes on either side of the mesenteric bed were removed by blunt dissection with a pair of curved forceps. Bleeding was controlled by lightly pressing the surrounding fatty tissue onto the connecting vessels. The intestine was then replaced and approximately 2 ml sterile 0.9% saline (with antibiotics if necessary) was added to prevent adhesions. The abdomen was closed with sutures and the animal was left for 5-6 weeks to allow the mesenteric lymphatic vessels to regenerate before immunization commenced.

6-8 weeks later their abdomens were re-opened and the small intestine again extracted as above and each of the 12-16 Peyer's patches were located. Between 10-15 μ l of a suspension of S. typhimurium (SL3261 BRD062) containing 1.25×10^7 CFU were injected into each Peyer's patch using a 27 gauge needle giving a total dose of approximately 1.25×10^8 in 0.1 ml per rat. Injection was aided by squeezing the region of the gut with the Peyer's patch so that the needle could be inserted just beneath the capsular membrane. Injection into the Peyer's patch could be verified by the swelling of the patch. Non-swelling was indicative of the needle piercing the gut wall and the contents being injected into the small intestine.

Group 2 consisted of rats which had not undergone lymphadenectomy. These rats were immunized via Peyer's patches as in group 1 above.

Both groups received secondary immunization 3-4 weeks after primary immunization. Secondary immunization was carried out intra-peritoneally with a total dose of 1.25×10^9 CFU of S. typhimurium (SL3261 BRD062).

2.3.2. Preparation of rat Y3 myeloma cells.

The fusion myeloma cell-line, Y3 Ag1.2.3, needs to be grown in spinner culture in order to produce cells competent for fusion. Starting a fresh spinner culture involved the unfreezing of an aliquot of Y3 Ag1.2.3 previously frozen down in liquid nitrogen at 4×10^6 cells in 95% FCS / 5% dimethyl sulphoxide (DMSO). The cells were washed twice in 10 mls Dulbecco's modified Eagle's medium (DMEM) with 10% FCS then transferred to 100 mls DMEM in a 20 mls *Bellco spinner flask. The flask was placed on a *Bellco magnetic stirrer at 37 °C in an incubator and allowed to stand for 2 days. The culture was then stirred at 160 rpm. The cells were maintained at a maximum of density of between 5×10^5 to 10^6 cells/ml by daily 4-fold dilution with fresh medium. Two days after commencement of stirring the cells were ready for fusion.

2.3.3. Removal and preparation of MLN for fusion.

The rat was sacrificed and the mesenteric lymph nodes (MLN) removed by blunt dissection with a pair of curved forceps. The MLN were then disaggregated by being

forced through a fine stainless-steel sieve using a spoon-headed spatula and collected into serum-free DMEM. The cells were centrifuged at 400 g for 2 minutes and washed twice in serum-free DMEM. A total leucocyte count was carried out and the cells resuspended to a concentration of $3-5 \times 10^7$ cells/ml.

2.3.4. Collection and preparation of EML for fusion.

Efferent mesenteric lymph (EML) was collected by canulation of the cisterna chyli, the principal lymphatic vessel draining the mesenteric lymphatic system into the thoracic duct. An immunized rat which had previously undergone mesenteric lymphadenectomy was anaesthetized with Sagatal (10mg/ml). The rat received approximately 0.9 ml intra-peritoneally which kept the rat unconscious for about 45 minutes. During the operation anaesthesia was continued with diethyl ether. The upper abdomen opened by a transverse incision. The cisterna chyli was located on the left side of the abdomen and fat deposits which surrounded it were removed using a blunt probe and cotton wool. Visualization can be aided by the injection of a small amount of Guerbert's patent blue V, a lymphography dye, into a Peyer's patch. The vessel was detached from the surrounding tissue such that sutures could be passed around it. The vessel was ligated 1-2 cm above where the last mesenteric lymphatic vessel joined. A small polyethylene canula (outer diameter 1 mm) with a curved end was flushed with DMEM containing 10% FCS and 10 IU heparin/ml to prevent clotting. The curved end of

the canula was then passed into the vessel just below the ligature so that lymph flowing from the mesenteric lymphatic system would enter the canula. The canula was then passed through the abdominal wall and secured with a metal clip. The end of the canula was examined for a few minutes to ensure that lymph is flowing from it and then was placed into a sterile foil capped 150 ml bottle containing 50 ml DMEM with 10% FCS and 10 IU heparin/ml. Approximately 5 ml of sterile 0.9% saline was placed in the peritoneal cavity to prevent adhesion and the abdominal wall was closed with sutures. The rat was then placed in a Bollman restraining cage and fed ad libitum with water containing 0.6% NaCl, 0.5% KCl and 2.5% glucose and food pellets.

2.3.5. Preparation of feeder cells for fusion cultures.

Fusion cultures were plated out on irradiated confluent monolayers of rat fibroblasts obtained from rat xiphoid cartilage. The feeder cell-line was prepared from xiphoid cartilages removed from 6 to 8 adult rats and chopped into small sections with a scalpel. These were then stirred for 75 minutes at room temperature in 15 mls DMEM containing 0.5% trypsin and 1% collagenase. The solution was filtered through sterile gauze and then spun at 400 g for 5 minutes. The pelleted cells are resuspended in DMEM with 10% FCS and plated out in sterile Nunc 80 cm² flat-bottomed flasks. The cells were capable of being passaged up to 20 passages before they lost fibroblast morphology.

Stocks of each passage were frozen down in liquid nitrogen in aliquots of $2-4 \times 10^6$ cells using 95% FCS/5% DMSO.

Twenty-four hours before a fusion feeder cell adherent monolayers were treated with 10 ml PBS pH 7.4 containing 0.04% disodium EDTA and 0.025% trypsin for 5 minutes at room temperature. The cells were decanted from the flask and washed by centrifugation for 2 minutes at 400 g. The cells were resuspended in 10 ml DMEM with 10% FCS in a sterile 25 ml plastic universal and irradiated with 3000 rads. The cells were then plated out at 1 ml per well containing 2×10^4 cells in 24-well Costar plates and incubated overnight at 37 °C in 5% carbon dioxide. The wells were aspirated before the fusion cultures were plated out.

2.3.6. Fusion protocol.

Y3 Ag1.2.3 myeloma cells were harvested in exponential growing phase and centrifuged in 50 ml aliquots at 400 g for 5 minutes. The cells were washed twice in serum-free DMEM and then resuspended at $1-2 \times 10^7$ cells/ml.

Approximately 10^8 lymphocytes were mixed with 5×10^7 Y3 Ag1.2.3 myeloma cells in a 10 ml sterile capped Falcon tube and pelleted by centrifugation at 400 g for 5 minutes. The supernatant medium was poured off and excess liquid on the walls of the tube were removed with a Pasteur pipette. The base of the tube was gently tapped to disrupt the cells and 1 ml of 50% polyethylene glycol

(PEG) was stirred into the cells over a period of 1 minute. Mixing was continued for a further minute by gently rolling the tube at an angle. The cells were then diluted with growth medium, first 2 mls over a period of 2 minutes then 5 mls over a period of 1 minute. The cells were then centrifuged at 400 g for 5 minutes and resuspended in 200 mls of HAT hybridoma selection medium plus 20% FCS (HAT medium consisted of DMEM with 13.6 ug/ml hypoxanthine, 0.19 ug/ml aminopterin and 3.8 ug/ml thymidine). The cells were plated out at 2 mls per well into 24-well plates containing seeded irradiated feeder cells. Cells were left for 7-10 days before being tested or fed fresh medium.

2.3.7. Screening of culture supernatants.

Fusion cultures were not fed until testing for specific antibody commences. After 7-10 days the fusion cultures were examined for the presence of hybridoma colonies and were then tested for specific antibody by radioimmunoassay (RIA) as outlined below. The hybridomas varied in their growth rates and it was necessary to test supernatants for specific antibody at different times.

2.3.8. Selection and maintenance of antibody secreting cells.

Wells containing specific antibody were fed after initial screening and examined under an inverted plate microscope. If more than one hybridoma colony is present then individual colonies are aspirated of the well bottom and transferred into separate well on fresh plates.

Secondary cultures undergo further testing and those producing specific antibody are cloned as below.

Cells were maintained on RPMI 1640 medium with 10% (v/v) FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 ug/ml streptomycin.

2.3.9. Preservation of cells for storage in liquid nitrogen.

Viable cells in exponential growth (1×10^6 cells/ml) were harvested from wells and centrifuged at 400 g for 10 minutes at 4 °C. Supernatant was discarded and the pellet resuspended in FCS containing 7.5% (v/v) DMSO. The cells were immediately transferred to sterile freezing ampoules and placed at -80 °C. The ampoules were left for 6 hours before transferring to the vapour phase of liquid nitrogen in a dewar.

Reconstitution consisted of rapidly thawing the vial then slowly adding 10 ml of serum-free RPMI 1640 medium in order to avoid osmotic shock. The cells were washed twice to remove the DMSO and then transferred into normal growth medium.

2.3.10. Cloning of hybridoma cells by limiting dilution.

Cloning consisted of a series of limiting dilutions in order to produce a cell-line derived from a single hybridoma cell. Cells were harvested by centrifugation at 400 g for 5 minutes. The cells were resuspended in 5 mls of fresh medium and a sample was counted under low-power using a Fuchs-Rosenthal counting chamber slide. Trypan blue was added as a vital stain. Only live cells were

counted. Following the estimation of cell number 2 ml of fresh medium was dispensed into 6 tubes, labelled 1 to 6. Tube 1 has a further 1 ml of fresh medium added. The cell suspension was then diluted so that 100 μ l (containing 7.5×10^3 cells) could be transferred to tube 1. Tube 1 was mixed thoroughly and a fresh pipette was used to transfer 1 ml from tube 1 to tube 2. This was repeated with all tubes to produce a serial dilution of 1 in 3. Eight ml of fresh medium was then added to produce a final volume of 10 ml in each tube.

Starting with tube 6 the cell suspensions were poured into a sterile trough and transferred into 48-wells of a 96-well plate with 200 μ l in each well. (Each 96-well plate had been pre-incubated with irradiated rat xiphoid cartilage feeder cells). This was repeated for each tube.

The result was 3 cloning plates with 100,33,11,3,1 and 0.3 cells per well. These were then left to grow up and were tested for specific antibody.

Wells containing specific antibody at the lowest concentration were grown up and transferred for further growth in 24-well plates and subsequent re-cloning. Selection of colonies from wells with 1 cell per well initial concentration were said to be cloned at 100% as statistically these colonies were derived from one cell.

Each cell line was cloned twice by limiting dilution at 100% each time.

2.3.11. Isotype determination.

Maximal growth medium from stable cell lines were tested for isotype specificity by radial immunodiffusion using antisera specific for rat IgA

2.4 SOLID PHASE RIA.

RIA was carried out based on the method of Pierce and Klinman (1976). RIA was used for the initial screening of hybridoma supernatants for antibody production and specificity. RIA was also used for characterization of cloned cell-lines together with ELISA described below. Antigens used in RIA included both the immunizing strain SL/BR and it's parent strain SL, together with other strains. Other antigens used include LT-B, purified LPS preparations and cellular preparations of the immunizing strain.

2.4.1. Adsorption of antigen to the solid phase.

Flexible polyvinyl chloride (PVC) 96-well "U"-bottomed microtitre plates (Dynatech, Billingham, Britain) were coated with 50 μ l of antigen preparation in PBS (pH 7.2) and incubated for 4 hours at room temperature followed by an overnight incubation at 4 °C. The optimal coating concentrations for each antigen were determined by titration with immune serum at varying dilutions in plates containing increasing concentrations of antigen solution. The lowest concentration of antigen which gave maximal binding of the anti-sera was chosen for subsequent screening.

2.4.2. Preparation of cytoplasmic and membrane fractions of Salmonella typhimurium SL3261 BRD026.

Strain SL/BR was grown up as above to log-phase of growth and then harvested in Tris-HCL (pH 6.8). The cells were pelleted by centrifugation at 1500 rpm for 15 minutes and resuspended in Tris-HCL (pH 6.8). This was repeated twice. The cells were then sonicated at maximum amplitude on ice for 15 minutes. To prevent heating sonication was carried out in periods of 30 seconds followed by 30 seconds rest.

The suspension was then centrifuged at 30,000 rpm for 30 minutes using a Sorvall ultracentrifuge. The supernatant was collected and formed the cytoplasmic fraction. The pellet was resuspended vigorously in Tris-HCL (pH 6.8) and recentrifuged at 1000 rpm for 10 minutes to remove any cellular debris. The supernatant was collected and formed the membrane fraction.

2.4.3. Blocking of unbound sites.

Sites capable of non-specific binding on antigen coated plates and control plates without antigen were blocked by incubating with 10% FCS in PBS (pH 7.2) for 2 hours at room temperature or overnight at 4 °C. The level of FCS in the blocking solution was determined using plates coated with various FCS solutions of differing concentration.

2.4.4. Addition of antibody.

Dilution series of Mab or polyclonal antisera for screening were prepared in 10% FCS/PBS (pH 7.2) and added to wells at a volume of 50 μ l for each dilution. Samples were added to antigen and control (no antigen) plates in replica with multiple replicates within each plate. Plates were incubated at room temperature for 2 hours or overnight at 4°C.

2.4.5. Iodination of antibody.

Goat anti-rat immunoglobulin and protein A were radioiodinated with Iodine 125 by the iodogen tube method of Fraker and Speck (1978). Iodogen (1,3,4,6-tetrachloro-3, 6 diphenylglucouril; Pierce and Warriner Ltd., Britain) was dissolved in dichloromethane (BDH) to give a concentration of 1 mg/ml. 25 μ l were then pipetted into a 2.0ml polypropylene Eppendorf tube and the solvent evaporated overnight. 50 μ l of a solution of 1mg/ml protein in PBS (pH 7.2) were added to the iodogen-coated tube and mixed by inversion. 10 μ l (1 mCi) of carrier-free sodium iodide 125 (Radio-chemical Centre, Amersham, Britain) was then added to the tube and incubated for 10 minutes with mixing every 60 seconds. The volume was then increased to 0.5 ml with 0.1% (w/v) BSA/PBS (pH 7.2) and then applied to a Sephadex G25 column (Medium grade; Pharmacia Ltd., Bucks., Britain) for desalting. The column had previously been washed with 25 ml 0.1% BSA/PBS (pH 7.2). Elution was carried out with 0.5 ml of 0.1% BSA/PBS (pH 7.2) and 0.5 ml fractions were collected. The

first fractions containing the peak of radioactivity as assessed by a gamma counter were pooled and stored with 0.02% sodium azide. To determine the specific bound activity 10 μ l of the radiolabelled antisera solution was added to 0.5 ml of 0.1% BSA/PBS. 0.5 ml of 10% TCA were then added to precipitate the protein. The solution was then centrifuged at 2000 rpm for 10 minutes and the radioactivity assessed in a Gamma-counter. The supernatant was then disposed of and the pellet was assessed for radioactivity. The specific bound activity was given by the activity of the pellet divided by the activity of the pellet plus the activity of the supernatant. This was expressed as a percentage.

2.4.6. Addition of Iodine 125-labelled antibody.

Radiolabelled antisera were diluted with 10% FCS/PBS (pH 7.2) to give 2×10^6 CPM/ml. 50 μ l of this dilution was added to each well. The plates were incubated for 2 hours at room temperature or overnight at 4 °C and then washed three times with 10% FCS/PBS (pH 7.2). The plates were cut up and each well was individually counted in a Gamma counter (LKB 1600 multigamma; LKB Instruments Inc., Turku, Finland) for 1 minute.

2.4.7. Determination of antibody titre.

The antibody titre was determined by serial dilution of the Mab maximal growth medium on plates coated with SL/BR as above. Samples were assayed in duplicate one each plate and each plate was duplicated, giving 4 sets of data for each Mab.

The titre was described as the 50% antibody binding titre (ABT50). This represented the titre at which each Mab gave 50% binding when compared with that of undiluted maximal growth medium.

2.5 ISOLATION OF MOLECULAR FORMS OF IGA MONOCLONAL ANTIBODIES.

A number of selected IgA-secreting hybridomas were shown to produce a range of molecular forms as seen on non-reduced SDS-PAGE. Maximal growth medium of two of these hybridomas was subjected to an isolation procedure based on Mestecky (1971) to purify these forms for further study.

Each pooled maximal growth medium was precipitated with two volumes of 27% (w/v) anhydrous sodium sulphate in distilled water at room temperature then incubated at 37°C for 2 hours. The precipitated globulin was then harvested by centrifugation for 10 minutes at 2000 g, room temperature. The globulin was then resuspended in PBS and re-precipitated as above. This was followed by extensive dialysis for 48 hours against PBS. IgA from the globulin fraction was then immunoprecipitated by a F(ab)2

preparation of goat anti-rat IgA antisera (Nordic Immunochemicals, Britain). The immunoprecipitate was collected by centrifugation at 2000 g for 10 minutes, room temperature, and redissolved in a glycine buffer (pH 2.75). The IgA fraction was then separated into its constituent molecular forms by molecular weight on a Sephacryl (S-200) column. Fractions were collected and their absorbance (280 nm) was measured. The absorbance profile for each Mab separation showed three peaks (A,B,C) between the void volume and the peak of the F(ab)₂ preparation with various low molecular weight digested proteins. The fractions of each peak (ie A, B and C) were pooled and dialysed extensively against PBS to restore the pH to 7.2. Each pooled preparation was then run on a non-reduced SDS-polyacrylamide gel to confirm the molecular weight and purity of each molecular form.

2.6 IN VITRO ADHERENCE ASSAY USING MADIN DARBY CANINE KIDNEY CELLS AND S. TYPHIMURIUM SL3261 BRD026.

Finlay et al. (1988) demonstrated that S. choleraesuis adhered to and invaded Madin Darby canine kidney (MDCK) cell monolayers in vitro. A similar in vitro assay was devised in this project for the immunizing strain S. typhimurium SL3261 BRD026 and MDCK cell monolayers in order to assess the ability of the rat Mabs to inhibit adherence, if any.

2.6.1. Growth and preparation of MDCK cells.

MDCK cells are a polarized epithelial cell line derived from the kidney of a cocker spaniel (ATCC, 1981). Cells were used between passage 97 and 121.

MDCK cells were grown in 225 cm² flat-bottomed flasks in Eagle's minimal essential medium (EMEM) with 5% FCS and 100 U/ml penicillin and 100 ug/ml streptomycin at 37 °C in 5% carbon dioxide. Cells were passage twice weekly with a split ratio of 1:4 with cells being frozen down at each passage. Monolayers took 3-4 days to achieve confluency in each flask and were removed by trypsinization using 0.025% (w/v) trypsin in a 2% solution of Versene.

Trypsinization involved the discarding of growth medium followed by the addition of 5 mls of the trypsinizing solution. This was dispersed around the flask bottom by gentle rocking for 30 seconds then discarded. A further 5 mls were added and the flask was sealed and incubated for 10 minutes at 37 °C, 5% carbon dioxide. The flask was then tapped to suspend the cells in the trypsin-versene solution and immediately diluted with 10 mls of fresh EMEM. The cells were decanted into a 50 ml centrifuge tube and further diluted with 40 mls of fresh EMEM. The cells were spun at 400 g for 10 minutes and then resuspended in fresh EMEM and transferred to a fresh flask or plated out in nine 96-well flat-bottomed polystyrene plates for each flask.

2.6.2. Assaying adherence of S. typhimurium SL/BR to MDCK monolayers.

MDCK cells which had been previously plated out in 96-well flat-bottomed polystyrene plates were examined for confluency prior to use in the assay. Only plates with confluent growth were used.

The medium was aspirated from the wells and replaced with EMEM plus 5% (v/v) FCS without antibiotics. This was repeated twice and the wells left empty.

S. typhimurium SL/BR was grown up as in Section 2.2.2 to exponential growth over 4 hours from static overnight cultures, harvested into PBS (pH 7.2) and adjusted to the required concentration.

SL/BR was added to the wells of the plates at 10^4 colony forming units (CFU's) per well in a total volume of 50 ul.

Each Mab was added in duplicate on each plate in a series of dilutions (neat, 1/4, 1/16, 1/32, 1/64, 1/256 and 1/1024) in a total volume of 50 ul EMEM plus 5% (v/v) FCS without antibiotics.

The wells were adjusted with EMEM plus 5% (v/v) FCS without antibiotics to a volume of 200 ul and incubated at 37°C and 5% carbon dioxide for 3 hours.

The assay was stopped by washing out the wells three times with ice cold PBS (pH 7.2).

2.6.3. Measurement of adherence of S. typhimurium SL/BR to MDCK monolayers by colony forming units.

Once the adherence assay was stopped, 50 ul of 0.025% (w/v) trypsin in a 2% solution of Versene was added to each well. The plates were then incubated for 5 minutes at 37°C , 5% carbon dioxide. The plates were then tapped to release the MDCK cells and each well was diluted with 150 ul of PBS (pH 7.2). Each well was then plated out at various dilutions on nutrient agar plates and left for 24 hours, after which the numbers of colonies were counted.

2.6.4. Measurement of adherence of S. typhimurium SL/BR to MDCK monolayers by ELISA.

After the adherence assay was stopped, 100 ul of a solution of 3:1 methanol/acetic acid was added to each well and left for 30 minutes after which the plates were washed with PBS (pH 7.2). 50 ul of SAM1/40B (maximal growth medium) was added to each well and incubated for 1 hour followed by washing with 10% FCS (v/v) in PBS (pH 7.2).

This was followed by 1 hour of blocking with 10% FCS (v/v) in PBS (pH 7.2) after which they were washed and 50 ul of goat anti-rat IgA conjugated with alkaline phosphatase at a dilution of 1/1000 was added to each well. The plates were incubated with 100 ul of p-nitrophenyl phosphate (PNP) in diethanolamine buffer for 45 minutes after which the reaction was stopped by the addition of 100 ul of 0.1 M sodium hydroxide (NaOH).

The plates were read in a Titertek Multiscan at 490 nm and the results expressed as ELISA units.

2.7 BACTERIAL CELL SURFACE HYDROPHOBICITY DETERMINATION.

Two methods were employed to estimate bacterial surface hydrophobicity as the initial method using octan-1-ol:water partitioning gave high degree of variation between samples. Hydrophobic interaction chromatography gave more reproducible results with less variation between samples.

2.7.1. Octan-1-ol method of determination.

One ml of a bacterial suspension containing 10^9 CFU/ml was added to 1 ml of octan-1-ol (Sigma, Britain) in a polypropylene tube and mixed for 2 minutes. The phases were allowed to separate at room temperature for 45 minutes after which the absorbance of the bacterial aqueous phase was measured at 540 nm. Control tubes containing 1 ml of PBS were used instead of octan-1-ol to measure the absorbance of the initial bacterial suspension.

The results were expressed as % hydrophobicity and were calculated as the absorbance of the initial bacterial suspension minus the absorbance after phase separation, divided by the initial bacterial suspension absorbance. The resulting fraction was converted to a percentage.

2.7.2. Hydrophobic interaction chromatography (HIC).

In order to assess the hydrophobicity of Mab treated and untreated bacteria was investigated by absorption and elution from octyl sepharose (Pharmacia, Britain).

Pasteur pipettes with siliconized glass wool in the funnel end and containing octyl sepharose were used as small separation columns. Equal volumes of octyl sepharose and 0.01 M phosphate buffer pH 6.8 containing 0.1 % (v/v) Triton X-100 were mixed and 2 ml were transferred into the Pasteur pipette. The column was equilibrated with the same buffer as were the bacterial suspensions. No aggregation of bacteria was seen before addition to the column.

One. ml of bacterial suspension containing 2×10^8 CFU was applied to the column and elution continued with 6 mls of equilibration buffer. The fraction of bacteria eluted was defined as hydrophilic and measured by absorbance at 540 nm. The percentage of the initial sample still absorbed to the column was estimated and the result expressed as the percentage hydrophobicity for the bacterial suspension.

2.8 BACTERIAL CELL SURFACE CHARGE DETERMINATION.

Net cell surface charge of bacteria and Mab-treated bacteria was measured by aqueous two-phase partitioning. In this system two polymers in aqueous solution form separate phases rich in each polymer due to the molecular interaction between molecules of the same polymer. In

this study an aqueous mixture of low concentrations of polyethylene glycol (PEG) and dextran results in a two-phase partition with a concentration of PEG in the top phase and dextran in the bottom. Bacteria added to these systems partition in a defined way dependant on their attraction for either PEG or dextran.

Addition of inorganic salts to these systems can also lead to non-equal partitioning of the one of the salt ions. Unequal partitioning of an ion preferentially into one phase with equal distribution of the other ion between the phases results in an electrical potential between the two phases. Bacteria added to such a system, if charged, will partition according not only to their preference for polymer but also according to the attraction of charge for each phase.

In this study two systems containing differing buffers were used to assess the net bacterial cell surface charge. Sodium chloride, an inorganic salt which partitions equally between PEG and dextran phases, was added as a buffer to one aqueous two-phase polymer system. Sodium sulphate was added to a second aqueous two-phase polymer system. The sulphate ions interact with the dextran polymers resulting in unequal partitioning with the bottom, dextran rich, phase becoming negatively charged compared with the upper PEG phase. The sodium chloride buffer system provides an non-charged system in which there is no electrical potential between the two phases. The sodium sulphate system results in an electrical potential between the phases providing a

charged system. By comparing the partition coefficients of treated and untreated bacteria in the two systems the relative net bacterial surface charge can be estimated (See Section 3.7.4).

2.8.1. Preparation of aqueous two-phase partitioning systems.

A basic two phase partition system containing 4.4% (w/w) polyethylene glycol (PEG) 6000 (molecular weight ??) and 6.2% (w/w) dextran T500 in 0.03% M Tris-HCl pH 7.0 was prepared from stock solutions of 20% (w/w) PEG 6000 in distilled water and 20% (w/w) dextran T500 in distilled water. The system was made up to a volume of 200 mls and left to separate overnight at 4 °C in a separating funnel. The bottom phase, rich in dextran, and the top phase, rich in PEG, were collected and stored separately at 4 °C.

In the uncharged system the initial partition system also contained sodium chloride (NaCl) to a final concentration of 0.1 M. In the charged system, sodium sulphate (NaSO₄) was added to a final concentration of 0.05M in the initial partition system before separation.

2.8.2. Aqueous two-phase partitioning.

To prepare phase systems for the partitioning assays 1 ml of the bottom phase and 1 ml of the top phase were pipetted into a test-tube. 0.1 ml of a bacterial suspension containing 1×10^9 CFU/ml was then added and the test-tube was vortexed for 1 minute. The test-tube was then left for 45 minutes to separate after which 0.5

ml aliquots were withdrawn from the top and bottom phase and the absorbance measured at 540 nm. Results were expressed as partition coefficients representing the concentration of the bacteria in the top phase divided by the concentration of the bacteria in the bottom phase.

Both uncharged and charged systems were carried out for each sample.

2.9 PHAGOCYTIC LUMINOL-DEPENDENT CHEMILUMINESCENCE.

Chemiluminescence (CL) occurs when phagocytic cells come into contact with particulate matter or microorganisms. Phagocytic cells are capable of releasing bactericidal oxygen species including superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen. These oxygen species produce CL by their transition of high energy electrons to lower energy states, the energy lost being emitted as light. The level of CL can be amplified by the addition of Luminol (5-amino-2,3-dihydro-1,4-phthalazine-dione, a cyclic hydrazide). Luminol-dependant chemiluminescence has been used to investigate the functioning of phagocytic cells and the use of antibodies for opsonization in phagocytic assays.

2.9.1. Growth and opsonization of bacteria.

N. meningitidis (type Y) was grown up overnight in a 5% CO₂ atmosphere at 37 °C on GC-agar. The plates were harvested in sterile saline and washed twice (centrifugation for 15 minutes at 2000 G, room temperature). The bacteria were resuspended in Hank's

balanced salt solution (HBSS, pH 7.3) buffered with 25 mM HEPES (N'-2-hydroxyethyl-piperazine N'-ethanesulphonic acid) without phenol red, at a concentration of 1×10^9 CFU/ml and left on ice. 500 μ l of bacterial suspension (1×10^9 CFU/ml) were added to 100 μ l of monoclonal antibody (maximal growth medium). The total volume was adjusted to 1 ml using HEPES-buffered HBSS and rotated for 30 minutes at 37 °C. The suspension was then centrifuged at 2000 G for 15 minutes and washed twice with HBSS. The opsonized bacteria were finally resuspended in 0.5 ml of HEPES-buffered HBSS and left on ice.

2.9.2. Preparation of human polymorphonuclear leucocytes.

Fresh blood was taken by venepuncture from healthy adult volunteers into 10 units/ml of preservative-free heparin and mixed for 10 minutes at room temperature to prevent coagglutination. The blood was mixed with 6% (w/v) dextran (MW 110,000) in 0.9% (w/v) sterile saline (Dextraven 110 injection B.P., Fisons PLC, Loughborough, Britain) 2 parts to 1 respectively, in a 50 ml syringe. The red cells were allowed to sediment under gravity for 45 minutes at room temperature. The upper layer of leucocyte-rich plasma was then decanted off through a bent 23 gauge needle. The fraction was then centrifuged at 160 G for 15 minutes and the pellet resuspended in 10 ml 0.83% (w/v) Tris-buffered ammonium chloride (pH 7.2) (Boyle's solution). The suspension was mixed for 10 minutes at 37 °C to lyse any residual cells. The cells were then washed twice in HEPES-buffered HBSS containing

0.1% FCS. The cells were left at 37 °C on a roller for 30 mins.

2.9.3. Microscopical phagocytosis assay.

Opsonized bacteria were diluted 1 in 100 in HEPES-buffered HBSS to give a concentration of 5×10^6 CFU/ml. 200 μ l of the suspension was incubated with an equal volume of PMN (2×10^6 cells/ml) for 15 minutes at 37 °C on a roller. A 100 μ l aliquot of this mixture was applied to a glass slide and cytocentrifuged (Shandon Southern Products Ltd, Cheshire, Britain) at 55 G for 5 minutes. The slide was air-dried, fixed in methanol and stained with acridine-orange (0.3 mg/ml). The number of neutrophils with ingested bacteria were counted using a fluorescence microscope and expressed as a percentage.

2.9.4. Chemiluminescence assay.

Luminol-dependant CL was measured using a photometer (Luminometer 1250, LKB Wallac, Sweden) at 37 °C. All reaction mixtures were kept at 37 °C in a Dri-block 08-3 (Techne Cambridge Ltd, Cambridge, Britain) during the assay. A stock solution of Luminol was dissolved in dimethyl sulphoxide (DMSO) and diluted in HBSS to give a final concentration of 2×10^{-5} mol/l. 900 μ l of the Luminol solution were added to a 3 ml polystyrene reaction vial containing 500 μ l of the PMN suspension prepared above. The mixture was transferred to the light proof chamber of the Luminometer, a background subtraction control was used to zero the instrument prior to the addition of the opsonized bacteria. 200 μ l of the

opsonized bacteria suspension prepared above was added to the reaction mixture and the maximum CL generated (in mV) was recorded after 15 seconds using the integral printer of the Luminometer. This was repeated for each reaction mixture every 2 minutes for a total of 90 minutes. Each reaction vial was kept covered from light at 37 °C in a Dri-block.

2.10 SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE).

Gels were run based on the methods described by Laemmli (1970). All gels were run at 10% acrylamide concentration with a width of 0.75 mm.

The running buffer was prepared with 6 g Tris-HCl, 28.8 g of glycine and 2 g of sodium dodecyl sulphate (SDS). These were added to 2 l of de-ionised distilled water. The sample buffer was stored as a double strength solution with 0.005% Bromophenol blue (BDH), 2.0% SDS, 20% Sucrose (BDH) in 0.08M Tris-HCl pH 6.8. Samples to be run on gels were mixed with equal volumes of the sample buffer prior to loading.

For non-reduced gels where dissociation of inter-chain bonds was not wanted the sample was then loaded. For reduced gels Mercaptoethanol to a concentration of 10% (v/v) was added to the sample following addition of the sample buffer. The sample was then boiled for 3 minutes at 100 °C. Once cool the sample was then loaded on the gel.

Gloves were worn at all times whilst casting, loading and handling gels to prevent contamination and for protection against the toxic effects of acrylamide and N,N'-methylene bisacrylamide.

Glass plates (20 cm x 20 cm) were washed thoroughly in water then dried and sprayed liberally with 70% methanol. The plates were then dried once more. Perspex spacers (0.75 mm width) were placed between the plates which were then clamped together. The plates were placed in the casting stand and sealed. The separating gel solution was poured into the mould leaving a 4-5 cm space at the top. Distilled water was layered on top of the gel to give a sharp interface and the gel left to polymerize for 30 minutes.

Once polymerized the water was poured off and a perspex comb (0.75 mm) was inserted between the glass plates to form wells for loading samples. The stacking gel solution was then poured on top of the separating gel and allowed to polymerize for a further 30 minutes.

The comb was gently removed and the wells dried with thin sections of filter paper. Using a P20 Gilson pipette each sample of 20 μ l was loaded into a well. Once all the samples were loaded the upper reservoir was assembled and the gel apparatus was connected to a LKB power pack. The gel was run at room temperature for 4 hours at 25 mA. The gel was stopped when the bromophenol dye front reached the bottom of the gel.

2.10.1. Coomassie blue staining of SDS-PAGE gels.

The coomassie blue stain contained 405 ml methanol, 520 ml distilled water and 0.125 g Coomassie blue dye. When staining glacial acetic acid was added to the stain at 7% (v/v). Gels were left in 100 ml of stain rocking overnight at room temperature. Destaining solution consisted of the same constituents as the coomassie blue stain above but without the dye. Gels were destained with four 100 ml changes of destain over 2 hours at room temperature.

2.10.2. Silver staining of SDS-PAGE gels.

The gel was placed in a fixing solution of 50% methanol and 10% acetic acid for 2 hours. The gel was then twice washed with 15% methanol for 10 minutes. The gel was then placed in a solution of 5% glutaraldehyde for 30 minutes to cross-link proteins. The gel was subsequently washed with 15% methanol as above. The gel was allowed to equilibrate for 2 hours in a solution of silver nitrate (1.9 g l^{-1}) in distilled water and then rinsed in distilled water for 30 seconds. The silver nitrate was reduced by a solution of sodium borohydride (NaBH_4 , 87.5 mg l^{-1}) and acetaldehyde (HCHO , 7.5 ml l^{-1}) in 0.75 M sodium hydroxide (NaOH) for 10 minutes, or until spots began to appear. The gels were dried or stored in distilled water.

3 . RESULTS .

3.1 STANDARDIZATION OF RADIOIMMUNOASSAYS (RIA'S) WITH VARIOUS ANTIGENS FOR SELECTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODY (MAB) SECRETING HYBRIDOMAS.

The initial stage in producing monoclonal antibodies consists of standardizing a screening assay for the selection of positive clones. The assay must be sensitive enough to detect low levels of antibody compared with anti-sera controls but not so sensitive as to detect non-specific binding.

The concentration and choice of blocking agents must also ensure low non-specific binding as this will represent the negative control.

Initial optimization of the radioimmunoassay's used for characterization used immune rabbit serum (IRS) raised against heat-killed SL/BR to optimize the coating conditions for the various antigens.

In each optimization experiment the IRS was titrated at three dilutions (10^{-3} , 10^{-4} and 10^{-5}) in an attempt to simulate the levels of antibody normally present in the primary wells of a fusion.

3.1.1. Determination of optimal coating conditions for Salmonella typhimurium SL3261 BRD026 (SL/BR) in a solid phase RIA.

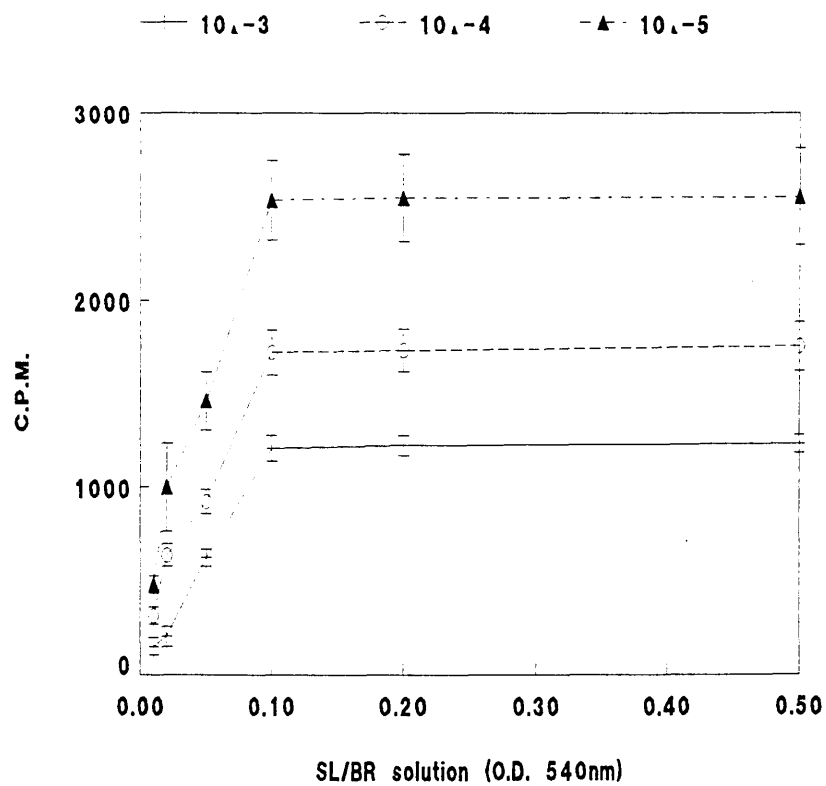
In order to select primary wells secreting specific antibody to the immunizing strain a solid phase radioimmunoassay was developed.

The initial screening of Mab supernatants was carried out with heat-killed Salmonella typhimurium SL3261 BRD026 (SL/BR), the immunizing strain, as the antigen and the use of IRS to optimize the coating conditions (see above, Section 3.1).

Plates were coated with a number of concentrations of heat-killed SL/BR as determined by optical density at 540 nm. The plates were then titrated with specific IRS. Non-specific binding sites were blocked using 10% (v/v) Foetal calf serum (FCS) in PBS. Wells blocked with 10% (v/v) FCS/PBS and not coated with SL/BR served as negative controls providing a record of non-specific background counts.

The results of these experiments are shown in Fig 4. From these results it was seen that coating the plates with a solution of SL/BR of optical density (O.D.) of 0.02 (540 nm) still enabled detection using all three dilutions of IRS. To ensure that the maximum coating conditions were achieved, enabling the highest levels of detection by IRS or Mab supernatant a coating solution of O.D. 0.1 (540 nm) was used in all subsequent assays.

Figure 4. Detection of S. typhimurium (SL/BR) at various concentrations in a solid phase radioimmunoassay using immune rabbit serum at 3 separate dilutions.



Coating solutions of greater concentration gave no increase in sensitivity of detection with any of the three dilutions.

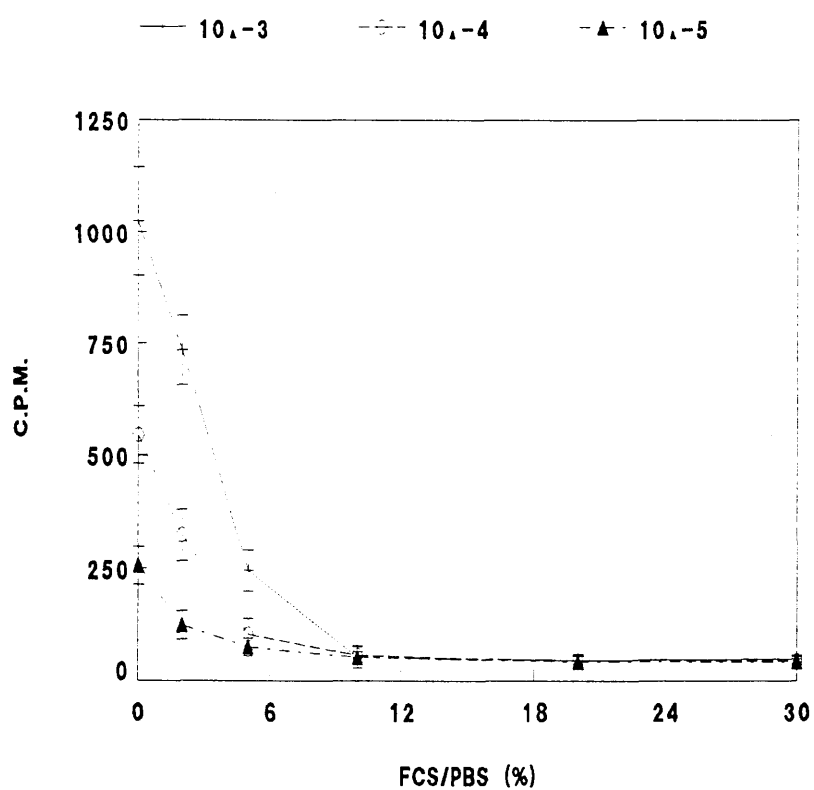
3.1.2. Determination of optimal blocking conditions using Foetal calf serum (FCS) in a solid phase RIA.

Foetal calf serum (FCS) was used as a blocking agent to reduce non-specific binding of Mab absorbing to the PVC microtitre wells of a plate in solid phase RIA. Initially, for standardization purposes, 20% (v/v) FCS/PBS was used as a blocking solution. For further assays the concentration of FCS was optimized.

Plates were blocked with differing concentrations of FCS/PBS and then titrated with IRS specific for SL/BR. Control plates blocked with PBS were used to measure absorption of IRS to PVC microtitre wells of the plates.

The results of these experiments are shown in Fig 5. It was seen that blocking the plates with a solution of FCS/PBS of 2% (v/v) still gave significant reductions in non-specific binding using all three dilutions of IRS. In order that the maximum blocking conditions were achieved to reduce the levels of non-specific binding by IRS or Mab supernatant a blocking solution of 10% (v/v) FCS/PBS was used in all subsequent assays both for lipopolysaccharide, bacteria and bacterial fractions. Blocking solutions of greater concentration gave no increase in reduction of non-specific binding with any of the three dilutions.

Figure 5. Blocking of non-specific binding by immune rabbit serum at 3 separate dilutions using various concentrations of foetal calf serum.



3.1.3. Determination of optimal coating conditions for cytoplasmic and membrane fractions of Salmonella typhimurium SL3261 BRD026 (SL/BR) in a solid phase RIA.

Subsequent screening of Mab supernatants was carried out with cytoplasmic and membrane fractions of Salmonella typhimurium SL3261 BRD026 (SL/BR), the immunizing strain, as antigens in a solid phase RIA and the use of immune rabbit serum (IRS) raised against heat-killed SL/BR to optimize the coating conditions (Figs 6 and 7).

Plates were coated with a number of concentrations of cytoplasmic and membrane fractions of SL/BR as determined by the protein content (eg 2ug protein/ml of cytoplasmic fraction) to enable comparison between the fractions. The plates were then titrated with specific IRS. Non-specific binding sites were blocked using 20% (v/v) FCS/PBS. Wells blocked with 10% (v/v) FCS/PBS and not coated with SL/BR served as negative controls as in previous assays.

The results of these experiments are shown in Figs 6 and 7. Coating the plates with a solution of the cytoplasmic fraction of SL/BR of protein concentration 2 ug/ml resulted in detection using all three dilutions of IRS. A solution of membrane fraction of SL/BR of protein concentration of 2 ug/ml for coating wells also resulted in detection by all three dilutions of IRS.

Maximum coating conditions enabling the highest levels of detection by IRS or Mab supernatant were found with a coating solution of protein concentration 10 ug/ml for the cytoplasmic fraction and a solution of protein concentration 10 ug/ml for the membrane fraction in the assays to characterize the specificity of the Mabs. Coating solutions of greater concentration for either fraction gave no increase in counts bound with any of the three dilutions and so would give no increase in the assay's sensitivity.

Figure 6. Detection of cytoplasmic fraction of S. typhimurium (SL/BR) at various concentrations in a solid phase radioimmunoassay using immune rabbit serum at 3 separate dilutions.

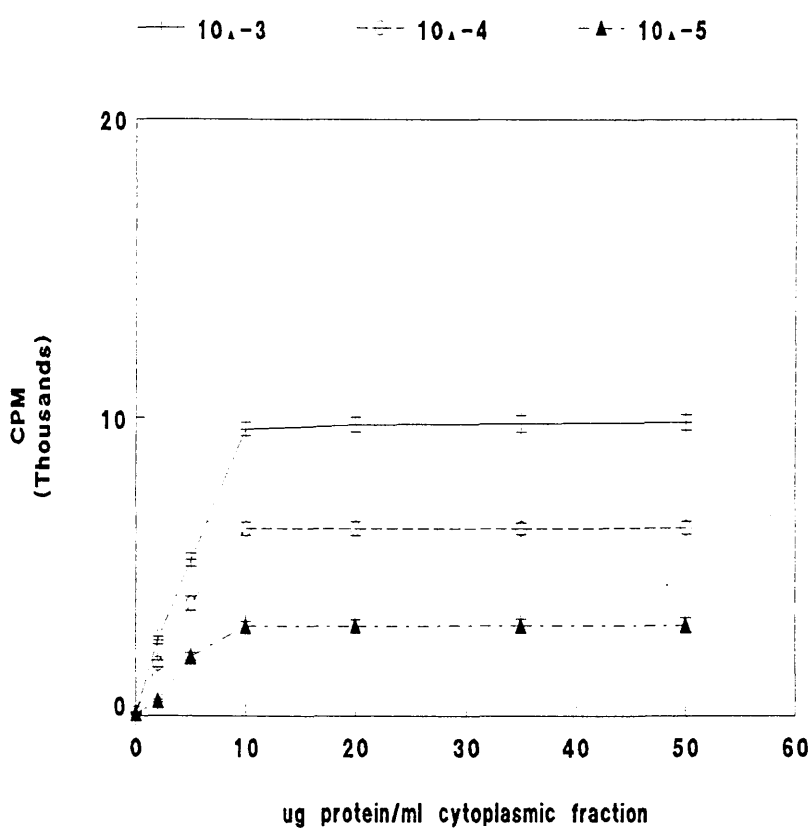
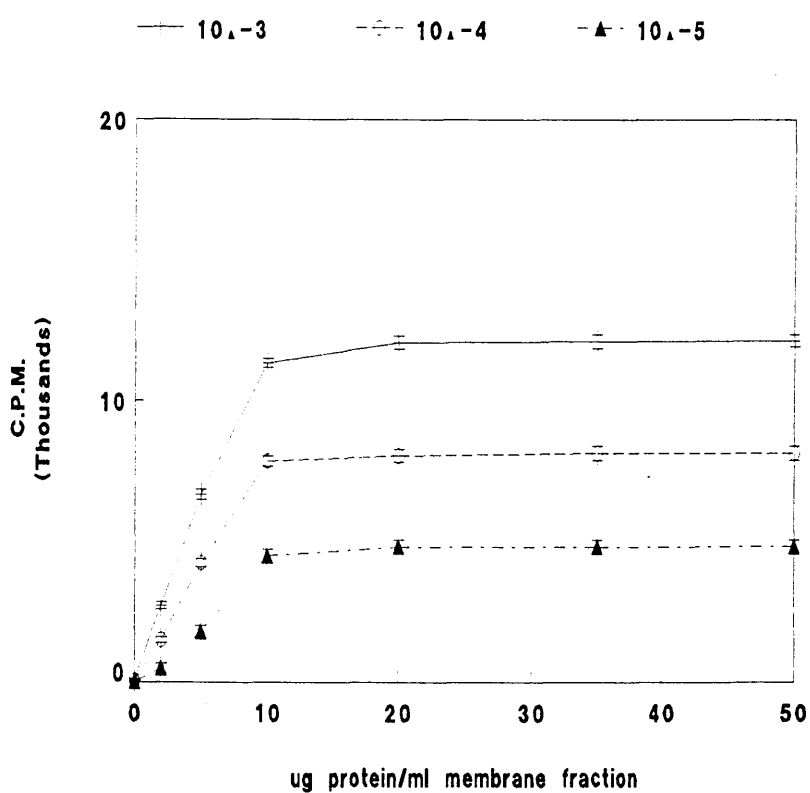


Figure 7. Detection of membrane fraction of S. typhimurium (SL/BR) at various concentrations in a solid phase radioimmunoassay using immune rabbit serum at 3 separate dilutions.



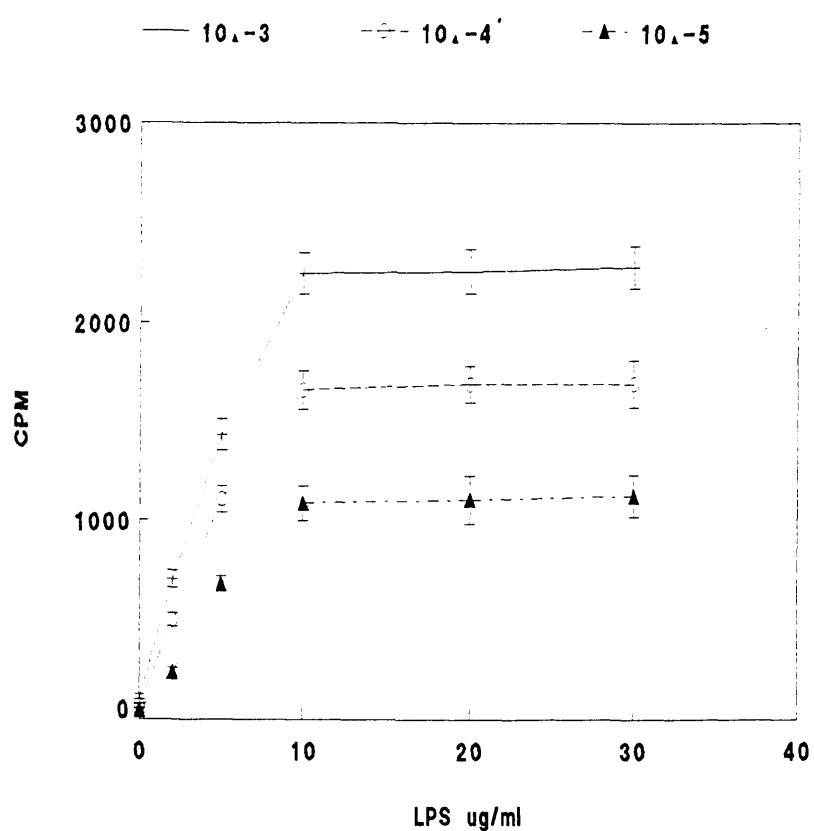
3.1.4. Determination of optimal coating conditions for Salmonella typhimurium (wild type) lipopolysaccharide (LPS) in solid phase radioimmunoassay (RIA).

Specificity of Mab supernatants for Salmonella typhimurium (wild type) lipopolysaccharide (LPS) was determined by using S. typhimurium (WT) LPS as antigen in a solid phase RIA. Immune rabbit serum (IRS) raised against heat-killed SL/BR was used to optimize the coating conditions (Fig 8).

Plates were coated with a number of concentrations of S. typhimurium (WT) LPS in PBS and were then titrated with IRS specific for heat-killed SL/BR. Non-specific binding sites were blocked using 10% (v/v) FCS/PBS. Wells blocked with 10% (v/v) FCS/PBS and not coated with S. typhimurium (WT) LPS served as negative controls as in previous assays.

The results of these experiments are shown in Fig 8. All three dilutions of IRS were capable of detecting S. typhimurium (WT) LPS on plates coated with a solution of 10 ug/ml LPS/PBS. In order to maximize coating conditions for detection by IRS or Mab supernatant a coating solution of 10 ug/ml S. typhimurium (WT) LPS/PBS was used

Figure 8. Detection of S. typhimurium (WT) lipopolysaccharide (LPS) at various concentrations in a solid phase radioimmunoassay using immune rabbit serum at 3 separate dilutions.



in the resulting assays to characterize the specificity of the Mabs. Coating solutions of greater concentration gave no increase in the assay's sensitivity. Further RIA using a range of LPS derived from other *Salmonella* species used the same coating conditions to allow comparison between the counts bound for each preparation.

3.1.5. Detection of Escherichia coli heat-labile enterotoxin, B sub-unit (LT-B) in heat-killed S. typhimurium SL3261 BRD026 (SL/BR), and SL/BR cytoplasmic and membrane fractions using solid phase radioimmunoassay (RIA).

It was initially hoped that Mab supernatants specific for Escherichia coli heat-labile enterotoxin, B sub-unit (LT-B) could be chosen by their differentiation between SL/BR, which produces LT-B, and SL, which does not. SL/BR had been shown to produce LT-B which was incorporated into it's outer-membrane (G. Dougan, personal communication). No qualitative differentiation between SL/BR and SL was found by initial screening of Mab supernatants (See Section 3.2.3). In order to assess the degree of expression of LT-B in heat-killed SL/BR and in it's cytoplasmic and membrane fractions plates coated as in the screening assays were probed with an immune rabbit serum (IRS) specific for LT-B (Table 1).

Table 1. Detection of LT-B in S. typhimurium SL3261
BRD026 (SL/BR), S. typhimurium SL3261 (SL) and
in cytoplasmic and membrane fractions of SL/BR
in radioimmunoassay using immune rabbit serum.

Antigen preparation	C.P.M	(Sd)
LT-B	2783.6	248.3
Membrane	1026.3	192.4
Cytoplasmic	864.4	203.6
SL	28.4	24.5
SL/BR	32.4	18.3

Heat-killed SL, which did not express LT-B, was used as a negative control whilst plates coated with GM1 ganglioside complexed with LT-B were used as positive controls. As the results show, LT-B specific IRS was unable to detect LT-B in the heat-killed SL/BR antigen preparation but did in both the SL/BR cytoplasmic and membrane fractions. There appeared to be a difference, although not significant, in the levels of LT-B in the two fractions with higher counts found in the membrane fraction compared with the cytoplasmic fraction. This may indicate that the LT-B is preferentially incorporated into the cell membrane as reported. Heat-killed SL/BR does not appear to contain antigenically intact LT-B. The heating involved in the killing of SL/BR is equivalent to that employed in destroying the toxicity of the intact LT. This may imply that the detoxification of the LT by heating results in the denaturing of the LT-B or LT-A sub-unit. The inability to detect LT-B in the heat-killed SL/BR but it's detection in the sonicated fractions of SL/BR may be due to denaturing of the LT-B sub-unit.

As the initial screening antigen (heat-killed SL/BR) did not contain antigenically intact LT-B this would explain the lack of LT-B specific Mabs selected.

3.2 CHARACTERIZATION AND CATEGORIZATION OF ISOTYPE AND SPECIFICITY OF MONOCLONAL ANTIBODIES (MABS).

Two sources of fusion tissue were used for four fusions which resulted in 32 cloned cell-lines secreting Mab specific for S. typhimurium SL3261 BRD026 (SL/BR).

The isotype of the Mabs was determined by radial immunodiffusion using isotype specific antisera. Twenty of the Mabs selected were of the IgA isotype, 11 were of the IgM isotype and only 1 Mab was of the IgG1 isotype.

3.2.1. Comparison of monoclonal antibodies (Mabs) by specificity and isotype.

Fig 9 presents the comparison of isotypes produced by using either mesenteric lymph nodes (MLN) or efferent mesenteric lymph (EML) as a source of lymphocytes for fusion. Although the numbers are small there appears to be little distinction between MLN and EML with regards to the isotype distribution of Mabs produced from each source. Approximately equal numbers of IgA and IgM were produced by each source with the single IgG1 antibody being produced by the EML.

Figure 9. Comparison of the isotypes of the selected monoclonal antibodies derived from mesenteric lymph node (MLN) tissue and efferent mesenteric lymph (EML)

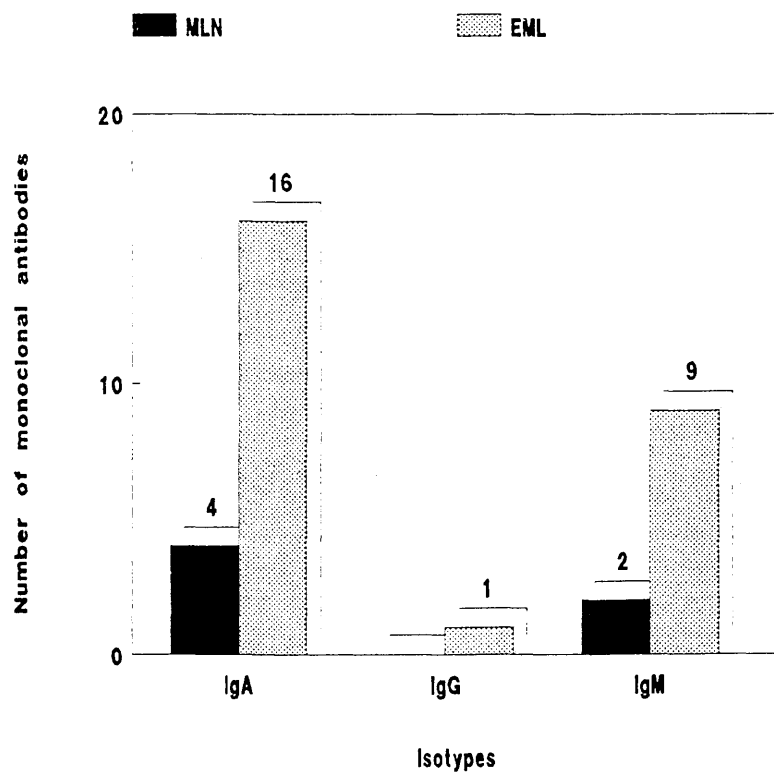
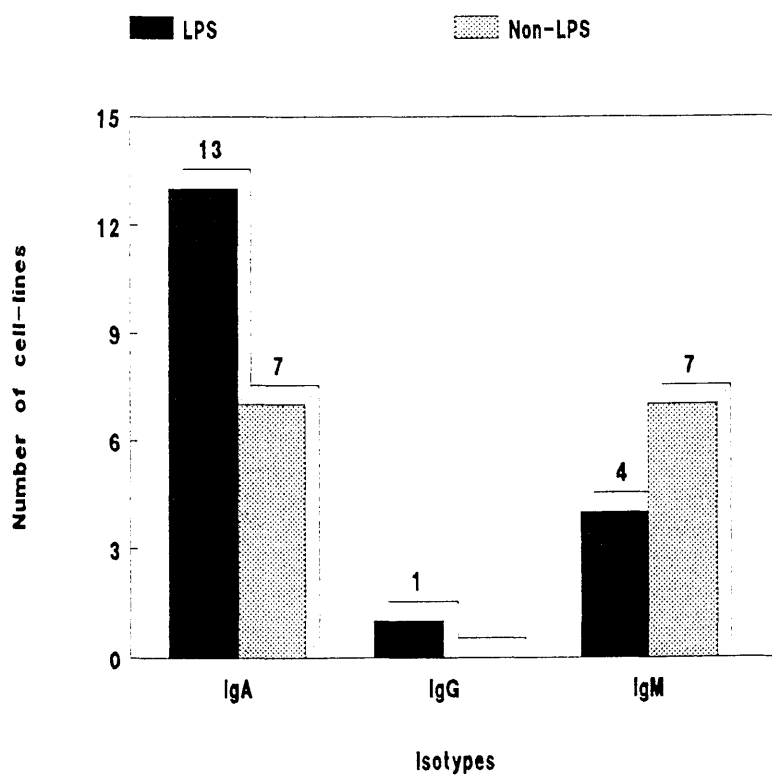


Figure 10. Comparison of the isotypes of the selected monoclonal antibodies directed against S. typhimurium (WT) lipopolysaccharide (LPS) and other antigens.



When characterized for specificity, the Mabs produced fell into two general groups based on their reactivity with S. typhimurium (WT) LPS. Fig 10 illustrates the distribution of isotypes of Mabs specific for S. typhimurium (WT) LPS. The numbers involved are again small but it can be seen that there is a distinct difference between the isotype distribution for Mabs which are LPS-specific and those which are not LPS-specific. Mabs which are not specific for LPS appear to be evenly shared by IgA and IgM isotypes but Mabs specific for LPS show a bias towards the IgA isotype. Approximately twice as many Mabs specific for LPS were of the IgA isotype as compared to those of IgM or IgG1 isotype.

3.2.2. Categorization of monoclonal antibodies.

The results of solid phase radioimmunoassays (RIAs) using various antigens to screen the 32 cell-lines secreting SL/BR specific Mab are presented in sections 3.2.3 to 3.2.4 and sections 3.2.6 to 3.2.8. Once completed the results of the screening RIAs formed the basis of a system of categorization based on isotype and reactivity for S. typhimurium (WT) lipopolysaccharide (LPS) and S. minnesota (WT) LPS. With this system the cell-lines were divided into 6 groups with the characteristics outlined in Table 2.

3.2.3. Specificity of monoclonal antibodies for S. typhimurium SL3261 BRD026 (SL/BR) and SL3261 (SL).

All Mabs were selected by their specificity for SL/BR. It was hoped that Mab supernatants specific for Escherichia coli heat-labile enterotoxin, B sub-unit (LT-B) could be chosen by their differentiation between SL/BR, which produces LT-B, and SL, which does not. No significant differentiation between SL/BR and SL was found by initial screening of Mab supernatants (See Section 3.1.5 above for further details).

Group 1 (IgA isotype, LPS specific, cross-reactive) contains 4 Mabs all with similar reactivity for SL/BR (Table 3). This similarity is also found in the reactivity with SL, however the Mabs appear to react less well with SL than SL/BR.

SL/BR and SL are isogenic with the exception that SL/BR carries the BRD026 plasmid coding for the LT-B sub-unit. As LT-B could not be detected in the antigen preparation of SL/BR it is unlikely that the presence of LT-B in SL/BR could account for the difference in reactivity for the Group 1 Mabs.

A difference in optimum coating conditions for the two strains would explain the disparity however optimum

Table 2. Group characteristics for the categorization of selected monoclonal antibodies.

Group 1.

IgA isotype, specific for S. typhimurium lipopolysaccharide (LPS), cross-reactive with S. minnesota LPS. (n = 4)

Group 2.

IgM isotype, specific for S. typhimurium lipopolysaccharide (LPS), cross-reactive with S. minnesota LPS. (n = 1)

Group 3.

IgA isotype, specific for S. typhimurium lipopolysaccharide (LPS), non cross-reactive with S. minnesota LPS. (n = 9)

Group 4.

Non IgA isotype, specific for S. typhimurium lipopolysaccharide (LPS), non cross-reactive with S. minnesota LPS. (n = 4)

Group 5.

IgA isotype, not specific for S. typhimurium lipopolysaccharide (LPS). (n = 7)

Group 6.

IgM isotype, not specific for S. typhimurium lipopolysaccharide (LPS). (n = 7)

(n = number of monoclonal antibodies within each group)

Table 3. Radioimmunoassay data for monoclonal antibodies of Groups 1 to 6 with S. typhimurium SL3261 BRD026 (SL/BR) and S. typhimurium SL3261 (SL) as antigen.

ANTIBODY	Binding (cpm +) to plates coated with					
	S. typhimurium			FCS		
	SL/BR		SL		(Control)	
		(Sd)		(Sd)		(Sd)
<u>Group 1</u>						
6/8/5D	6229.6	260.6	2373.6	211.8	38.4	21.6
6/8/13E	6175.9	246.7	5682.2	349.5	72.6	23.1
6/8/81C	6672.6	185.6	2663.8	364.1	60.9	17.8
6/8/13B	1142.8	185.8	941.5	55.3	163.8	41.7
<u>Group 2</u>						
7/8/39C	452.3	56.7	568.3	35.4	50.7	14.5
<u>Group 3</u>						
PIP 18	10154.5	475.6	2528.6	121.5	59.8	20.1
6/8/54A	373.3	83.9	562.3	53.8	152.7	43.6
PIP 2	6559.2	238.1	5667.8	220.1	54.7	24.2
7/8/52C	362.8	92.9	352.3	89.3	18.2	16.7
6/8/16	7237.1	341.8	7821.3	256.3	94.6	13.9
6/8/33C	4989.3	231.9	4561.3	185.3	41.7	17.5
6/8/42A	3249.3	178.2	3964.2	296.3	98.5	22.2
6/8/81B	4231.8	188.9	4523.9	133.9	42.5	12.7
6/8/25	330.9	67.8	325.6	88.8	57.5	19.4
<u>Group 4</u>						
PIP 13	4225.5	251.3	4001.7	211.3	63.8	23.4
7/8/18	5674.9	156.3	5502.5	238.3	44.5	21.9
6/8/53B	5211.9	189.1	5115.6	151.7	104.1	18.9
7/8/64	678.9	134.9	728.9	143.2	215.8	35.1
<u>Group 5</u>						
SAM1/40B	373.4	43.9	296.4	29.3	54.3	20.6
PIP 10	278.3	26.9	256.3	27.7	48.5	17.2
7/8/72A	543.2	47.9	452.3	45.3	39.1	13.5
7/8/72B	231.2	59.3	256.3	31.4	52.6	21.1
6/8/26C	349.5	26.3	342.6	28.1	156.9	38.2
6/8/33B	572.3	49.1	589.4	52.1	139.3	35.9
6/8/33A	330.9	21.9	325.6	25.2	57.5	24.1
<u>Group 6</u>						
6/8/54D	3083.8	349.2	1966.2	298.4	34.7	15.2
6/8/13C	202.6	54.8	324.7	45.7	39.6	10.8
6/8/13A	256.3	34.9	234.8	36.7	25.8	12.5
6/8/81D	824.7	72.1	1339.2	56.8	130.4	24.2
PIP 26	289.1	27.9	301.4	37.2	90.7	22.1
7/8/21	237.8	30.1	356.7	28.1	22.4	11.8
7/8/58B	362.7	34.2	402.5	29.9	106.2	28.3

coating conditions were the same for both strains. A possible explanation may be the presence of ampicillin-resistance genes carried on the BRD026 plasmid which allow for it's selection. Although this is the only difference between the two strains it is unclear how this could effect the binding of the Mabs which have been shown to be LPS-specific.

Group 2 (IgM isotype, LPS specific, cross-reactive) contains only one Mab which does not appear to have any distinction in it's reactivity with either strain (Table 3).

Group 3 (IgA isotype, LPS specific, non cross-reactive) contains 9 Mabs. Eight of the Mabs each exhibit similar reactivities with SL/BR and SL (Table 3) but one (PIP18) shows far greater reactivity with SL/BR than SL. The other Mabs also show increased reactivity to SL/BR compared with SL although the differences are not statistically significant.

Group 4 (non IgA isotype, LPS specific, non cross-reactive) has four Mabs, 3 of the IgM isotype and 1 IgG1 (Table 3). No significant difference is seen in their reactivity with SL/BR and SL.

Mabs from groups 1 to 4 are all specific for LPS. Group 5 (IgA isotype, non LPS specific) Mabs give much lower reactivities both with SL/BR and SL than the vast majority of Mabs within groups 1 to 4 (Table 3). This may well reflect the fact that LPS is the immunodominant antigen of Salmonella species. Selected mabs which are not LPS specific may be specific for antigens present in

low density within SL/BR or which are only weakly antigenic. This would result in lower reactivities due to a lack of antigen in the assay. Reactivity with SL/BR is higher for the group than with SL.

Group 6 also shows the same low reactivity as group 5 (Table 3). However one Mab (6/8/54D) shows significantly higher reactivities with both SL/BR and SL.

3.2.4. Specificity of monoclonal antibodies for S. typhimurium SL3261 BRD026 (SL/BR) cytoplasmic and membrane fractions.

Fractionation of SL/BR into cytoplasmic and membrane preparations was intended to aid in distinguishing those Mabs which were specific for surface structures on SL/BR. Differentiation was only seen in a quantitative rather than a qualitative manner. This overlap in reactivity with the two preparations was subsequently explained by SDS-PAGE. Inspection of samples of the preparations on gels stained with silver and with coomassie blue revealed a high level of cross-contamination between the cytoplasmic and membrane fractions (Fig 11).

Group 1 (IgA isotype, LPS specific, cross-reactive) all show similar reactivity for cytoplasmic fraction (Table 4). This similarity is also found in the reactivity to membrane fraction. The Mabs appear to react less well with membrane fraction than cytoplasmic fraction.

Figure 11. SDS-PAGE of S. typhimurium SL3261 BRD026
(SL/BR) cytoplasmic and membrane fractions
developed with Coomassie blue and Silver stain.

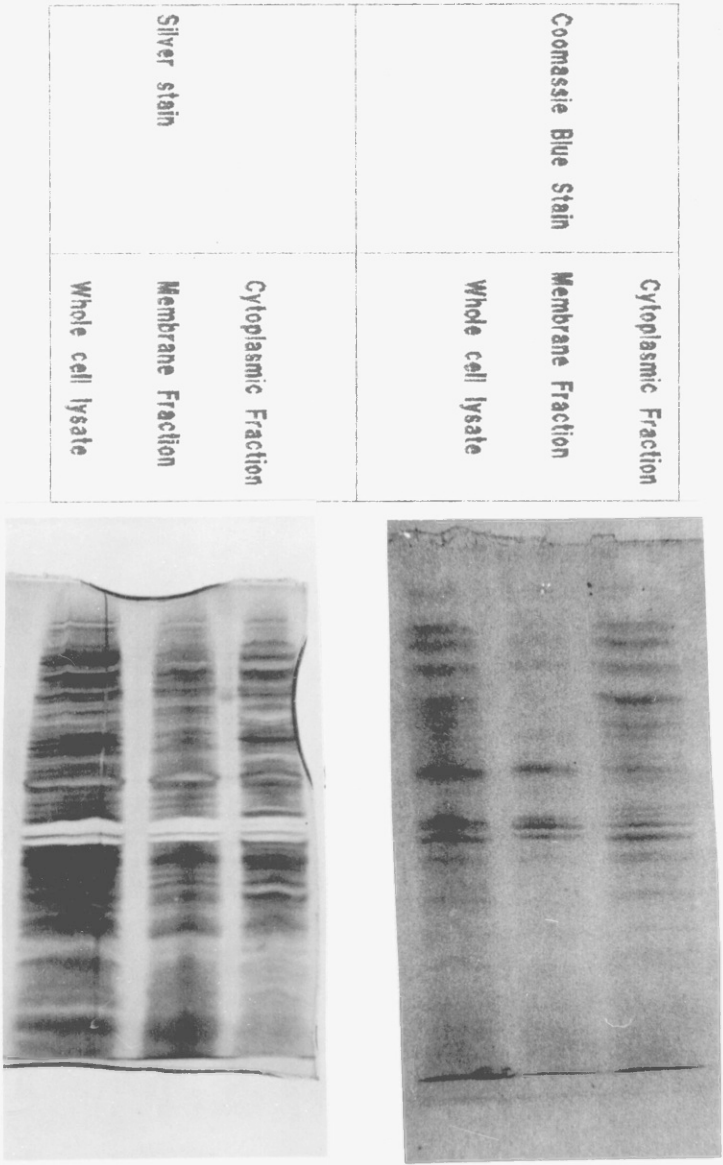


Table 4. Radioimmunoassay data for monoclonal antibodies of Groups 1 to 6 with S. typhimurium SL3261 BRD026 (SL/BR) cytoplasmic and membrane fractions as antigen.

ANTIBODY	Binding (cpm +) to plates coated with					
	S. typhimurium (SL/BR) fraction				FCS	
	Cytoplasmic		Membrane		(Control)	
		(Sd)		(Sd)		(Sd)
<u>Group 1</u>						
6/8/5D	5375.2	156.2	4677.1	563.5	38.4	21.6
6/8/13E	5752.8	78.9	4774.1	984.7	72.6	23.1
6/8/81C	6003.3	98.4	3283.9	285.2	60.9	17.8
6/8/13B	2465.3	121.2	1820.2	301.3	163.8	41.7
<u>Group 2</u>						
7/8/39C	412.9	48.6	516.7	36.2	50.7	14.5
<u>Group 3</u>						
PIP 18	6487.1	201.8	4765.2	189.6	59.8	20.1
6/8/54A	1754.6	104.5	1075.2	87.6	152.7	43.6
PIP 2	5767.7	235.6	5034.5	178.1	54.7	24.2
7/8/52C	317.7	35.6	485.1	25.6	18.2	6.7
6/8/16	6292.4	157.9	6503.8	156.3	94.6	13.9
6/8/33C	5380.8	231.4	5504.5	119.2	41.7	17.5
6/8/42A	3472.3	188.8	3678.2	136.3	98.5	22.2
6/8/81B	3489.2	267.8	3567.4	178.6	42.5	12.7
6/8/25	536.9	53.1	428.7	56.7	57.5	19.4
<u>Group 4</u>						
PIP 13	1922.3	201.9	2034.2	204.2	63.8	23.4
7/8/18	6607.9	321.9	6125.7	172.3	44.5	21.9
6/8/53B	6269.5	198.3	6003.9	231.5	104.1	18.9
7/8/64	1080.9	156.3	983.2	136	215.8	35.1
<u>Group 5</u>						
SAM1/40B	1207.9	136.2	829.8	104.2	54.3	20.6
PIP 10	560.3	78.4	467.9	67.3	48.5	17.2
7/8/72A	1685.5	129.1	1137.2	192.3	39.1	13.5
7/8/72B	478.1	52.9	362.4	112.9	52.6	21.1
6/8/26C	1505.5	199.1	1101.1	134.7	156.9	38.2
6/8/33B	4270.7	231.9	3782.9	145.7	139.3	35.9
6/8/33A	536.9	89.9	428.7	92.3	57.5	24.1
<u>Group 6</u>						
6/8/54D	2969.5	185.6	264.8	63.8	34.7	15.2
6/8/13C	683.9	69.5	237.6	28.4	39.6	10.8
6/8/13A	844.5	70.9	301.6	31.8	25.8	12.5
6/8/81D	2750.3	282.3	876.9	85.4	130.4	24.2
PIP 26	1010.7	113.5	589.9	59.2	90.7	22.1
7/8/21	482.9	59.5	345.2	45.7	22.4	11.8
7/8/58B	402.4	26.9	378.9	34.8	106.2	28.3

Group 2 (IgM isotype, LPS specific, cross-reactive) contains only one Mab which did not appear to distinguish in it's reactivity between either fraction (Table 4).

Group 3 (IgA isotype, LPS specific, non cross-reactive) contains 9 Mabs, all of which exhibited similar reactivities with cytoplasmic and membrane fractions (Table 4). The Mabs showed increased reactivity to cytoplasmic fraction compared with membrane fraction although the differences were not statistically significant.

Group 4 (non IgA isotype, LPS specific, non cross-reactive) has four Mabs, 3 of the IgM isotype and 1 IgG1. No significant difference is seen in their reactivity with cytoplasmic fraction and membrane fraction (Table 4).

Group 5 (IgA isotype, non LPS specific) Mabs gave much higher reactivities both with cytoplasmic fraction and membrane fraction than with SL/BR or SL (Table 4).

These selected Mabs may be specific for antigens present within the cytoplasm or which are only weakly antigenic on the surface membranes due to being covered by LPS. The sonication involved in the antigen preparation of the cytoplasmic and membrane fraction could result in the release of these antigens allowing greater access for specific Mabs. Lower reactivities may also be due in part to a lower level of specific antigen in the assay compared with other antigens such as LPS.

Further explanations could be found in the method of preparation of the antigens which did not include the

denaturing step involved in the heat-killing of the SL/BR and SL. Reactivity with both the cytoplasmic fraction and the membrane fraction is the same within this group.

Group 6 however showed a much higher reactivity for the cytoplasmic fraction than for the membrane fraction (Table 4). The group would appear to be unique from the other groups being the only one to show a significant difference in reactivity between the two antigen preparations.

3.2.5. Agglutination by monoclonal antibodies of S. typhimurium SL3261 BRD026 (SL/BR) and SL3261 (SL).

All monoclonal antibodies in Group 1 (IgA isotype, LPS specific, cross-reactive) demonstrate comparable agglutination of SL/BR (modal titre: 1024) (Table 5) and SL (modal titre: 256) (Table 6). As a group the Mabs appear to agglutinate SL/BR more efficiently than SL.

Group 2 (IgM isotype, LPS specific, cross-reactive) contains only one Mab which did not appear to distinguish in it's agglutination between either giving only a very low titre of agglutination (titre: 16) (Table 5 and 6).

Group 3 (IgA isotype, LPS specific, non cross-reactive) contains 9 Mabs, all of which exhibited similar agglutination titres with SL/BR and SL. The Mabs as a group showed increased agglutination to SL/BR (modal titre: 1024) (Table 5) compared with SL (modal titre: 256) (Table 6) but not to a significant degree.

Group 4 (non IgA isotype, LPS specific, non cross-reactive) has four Mabs, 3 of the IgM isotype and 1 IgG1.

Table 5. Agglutination of S. typhimurium SL3261 BRD026
(SL/BR) by monoclonal antibodies of Groups 1 to
6.

	Agglutination titre						
	1	4	16	64	256	1024	2048
<u>Group 1</u>							
6/8/5D	+++	+++	+++	++	++	+	-
6/8/13E	+++	+++	++	++	++	+	-
6/8/81C	+++	+++	+++	++	++	+	-
6/8/13B	+++	+++	++	++	++	+	-
<u>Group 2</u>							
7/8/39C	++	++	+	-	-	-	-
<u>Group 3</u>							
PIP 18	+++	+++	+++	+++	+++	++	-
6/8/54A	+++	+++	++	+	-	-	-
PIP 2	+++	+++	+++	+++	+++	++	-
7/8/52C	+++	++	+	-	-	-	-
6/8/16	+++	+++	+++	++	++	-	-
6/8/33C	+++	+++	+++	++	++	+	-
6/8/42A	+++	+++	+++	++	++	+	-
6/8/81B	+++	+++	+++	+++	+++	++	-
6/8/25	+++	+++	+++	++	++	-	-
<u>Group 4</u>							
PIP 13	+++	+++	+++	+++	++	+	-
7/8/18	+++	+++	+++	+++	++	+	-
6/8/53B	+++	+++	+++	+++	+++	++	-
7/8/64	-	-	-	-	-	-	-
<u>Group 5</u>							
SAM1/40B	+	-	-	-	-	-	-
PIP 10	+	-	-	-	-	-	-
7/8/72A	+	+	-	-	-	-	-
7/8/72B	+	+	-	-	-	-	-
6/8/26C	+	+	-	-	-	-	-
6/8/33B	+	+	-	-	-	-	-
6/8/33A	+	-	-	-	-	-	-
<u>Group 6</u>							
6/8/54D	+	+	-	-	-	-	-
6/8/13C	+	+	-	-	-	-	-
6/8/13A	+	+	-	-	-	-	-
6/8/81D	+	+	-	-	-	-	-
PIP 26	+	-	-	-	-	-	-
7/8/21	+	-	-	-	-	-	-
7/8/58B	+	-	-	-	-	-	-

Table 6. Agglutination of S. typhimurium SL3261 (SL) by
monoclonal antibodies of Groups 1 to 6.

	Agglutination titre						
	1	4	16	64	256	1024	2048
<u>Group 1</u>							
6/8/5D	+++	+++	+++	++	+	-	-
6/8/13E	+++	+++	++	++	+	-	-
6/8/81C	+++	+++	+++	++	+	-	-
6/8/13B	+++	+++	++	++	+	-	-
<u>Group 2</u>							
7/8/39C	++	++	+	-	-	-	-
<u>Group 3</u>							
PIP 18	+++	+++	+++	+++	+++	++	-
6/8/54A	+++	+++	++	+	-	-	-
PIP 2	+++	+++	+++	+++	++	-	-
7/8/52C	+++	++	+	-	-	-	-
6/8/16	+++	+++	+++	++	-	-	-
6/8/33C	+++	+++	+++	++	+	-	-
6/8/42A	+++	+++	+++	++	++	-	-
6/8/81B	+++	+++	+++	+++	+	-	-
6/8/25	+++	+++	+++	++	++	-	-
<u>Group 4</u>							
PIP 13	+++	+++	+++	+++	++	+	-
7/8/18	+++	+++	+++	+++	++	+	-
6/8/53B	+++	+++	+++	+++	+++	++	-
7/8/64	-	-	-	-	-	-	-
<u>Group 5</u>							
SAM1/40B	+	-	-	-	-	-	-
PIP 10	+	-	-	-	-	-	-
7/8/72A	+	+	-	-	-	-	-
7/8/72B	+	+	-	-	-	-	-
6/8/26C	+	+	-	-	-	-	-
6/8/33B	+	+	-	-	-	-	-
6/8/33A	+	-	-	-	-	-	-
<u>Group 6</u>							
6/8/54D	+	+	-	-	-	-	-
6/8/13C	+	+	-	-	-	-	-
6/8/13A	+	+	-	-	-	-	-
6/8/81D	+	+	-	-	-	-	-
PIP 26	+	-	-	-	-	-	-
7/8/21	+	-	-	-	-	-	-
7/8/58B	+	-	-	-	-	-	-

Only the 3 IgM Mabs were capable of agglutinating both SL/BR and SL with no significant difference seen in their agglutination of SL/BR (modal titre: 1024) (Table 5) or SL (modal titre: 1024) (Table 6). The IgG1 Mab showed no agglutination of either SL/BR or SL.

Group 5 (IgA isotype, non LPS specific) Mabs gave much lower agglutination titres with SL/BR (modal titre: 4) (Table 5) and SL (modal titre: 4) (Table 6) than any of the LPS-specific Mabs with much greater variation within the group than in the previous RIA data. Agglutination of both SL/BR and SL was the same for each Mab.

Group 6 also showed a much lower agglutination of SL/BR and SL (SL/BR modal titre: 4, SL modal titre: 4) (Table 5 and 6) than the LPS-specific Mabs of Groups 1 to 4. As with Group 5 there was also significant differences in agglutination within the group but little variation between SL/BR and SL for each Mab.

As already suggested in Section 3.2.4 above the Mabs of Groups 5 and 6 may be specific for intracellular antigens not present on the surface or for structures which are on the surface of the bacteria but only weakly antigenic. Agglutination would require an antibody directed against a surface antigen. The comparatively poor agglutination of both SL/BR and SL by the Mabs of Group 5 and 6 would suggest that they may not be directed against surface structures. The low levels of agglutination may be due to non-specific aggregation.

3.2.6. Specificity of monoclonal antibodies for S. typhimurium (Wild type) lipopolysaccharide (LPS) : Groups 1 to 4.

The selected Mabs were found to fall into two general groups based on their reactivity with S. typhimurium (WT) LPS. Group categorization of the Mabs was carried out using LPS-specificity as a criterion. Groups 1 to 4 were all LPS specific whilst groups 5 and 6 were not.

All group 1 (IgA isotype, LPS specific, cross-reactive) Mabs displayed similar reactivity for S. typhimurium (WT) LPS (Table 7). Group 2 (IgM isotype, LPS specific, cross-reactive) contains only one Mab which although LPS-specific gave far lower counts than group 1 Mabs (Table 7). Group 3 (IgA isotype, LPS specific, non cross-reactive) Mabs showed a range of reactivities from counts comparable with that of the group 1 Mabs to those comparable with that of the group 2 Mabs (Table 7). Group 4 (non IgA isotype, LPS specific, non cross-reactive) had four Mabs, 3 of the IgM isotype and 1 IgG1. Their reactivity was comparable to those of group 1 with the exception of the IgG1 Mab (7/8/64) which was significantly lower (Table 7).

Specificity for S. typhimurium (WT) LPS based on the results of the RIA was confirmed by autoradiography of Western blots from SDS-PAGE with S. typhimurium (WT) LPS probed by each Mab (see Fig 12).

Figure 12. Autoradiograph of S. typhimurium (WT) lipopolysaccharide (LPS) on SDS-PAGE probed with monoclonal antibodies (Group 1 to 4) and developed with goat anti-rat immunoglobulin conjugated with Iodine¹²⁵.

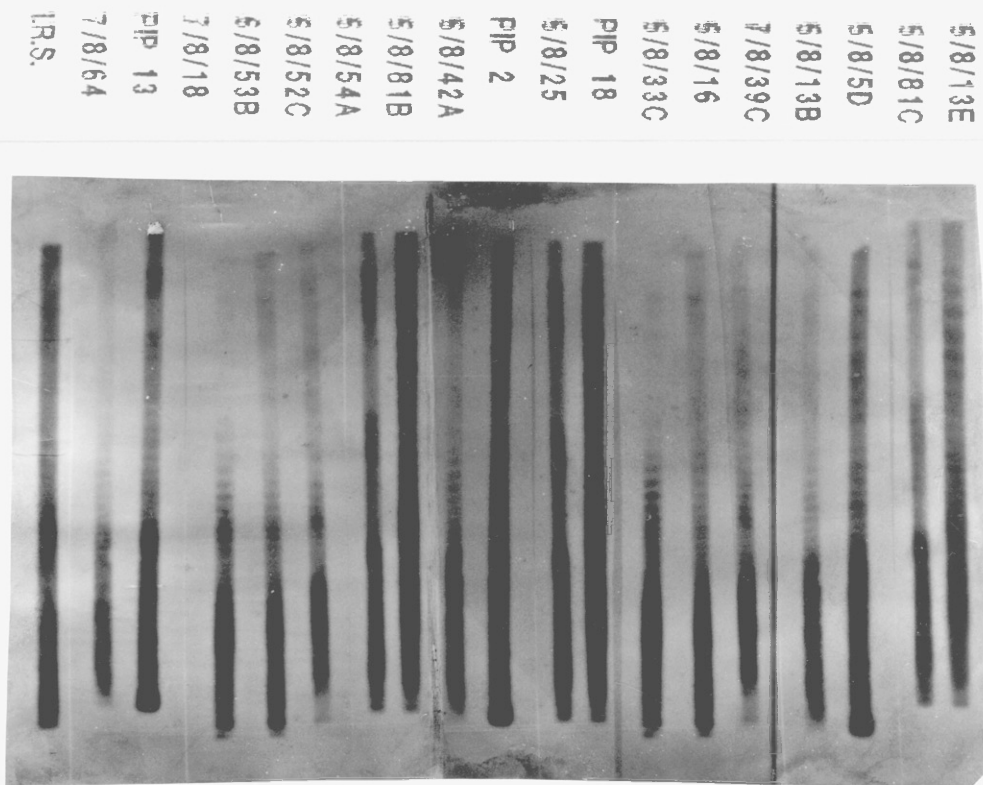


Table 7. Radioimmunoassay data for monoclonal antibodies of Groups 1 to 4 with S. typhimurium (WT) lipopolysaccharide (LPS) and S. minnesota (WT) LPS as antigen.

ANTIBODY	Binding (cpm +) to plates coated with					
	Lipopolysaccharide (LPS)			FCS		
	<u>S. typhimurium</u>	<u>S. minnesota</u>		(Control)		
	(Sd)	(Sd)		(Sd)		
<u>Group 1</u>						
6/8/5D	3680.3	326.1	2386.3	276.8	38.4	21.6
6/8/13E	4045	267.4	847.3	126.1	72.6	23.1
6/8/81C	3978.3	223.9	1575.6	287.3	60.9	17.8
6/8/13B	3459.9	342.1	651.6	98.7	163.8	41.7
<u>Group 2</u>						
7/8/39C	328.9	26.4	265.3	56.3	50.7	36.2
<u>Group 3</u>						
PIP 18	3680.9	132.9	117.2	27.9	59.8	20.1
6/8/54A	723.4	35.7	78.6	13.8	152.7	43.6
PIP 2	2928.5	221.9	91.7	34.6	54.7	24.2
7/8/52C	216.4	26.1	27.6	12.7	18.2	6.7
6/8/16	4309.4	201.8	100.1	23.7	94.6	13.9
6/8/33C	3901.9	123.6	63.8	27.4	41.7	17.5
6/8/42A	2553.7	153.9	94.2	32.3	98.5	22.2
6/8/81B	2413.2	133.9	37.2	17.3	42.5	12.7
6/8/25	3204	178.8	157.2	79.4	57.5	19.4
<u>Group 4</u>						
PIP 13	1444.7	118.4	100.2	23.4	63.8	23.4
7/8/18	3914.5	234.1	57.8	21.9	44.5	21.9
6/8/53B	4738.1	156.3	82.6	18.9	104.1	18.9
7/8/64	875.6	78.3	28.4	35.1	215.8	35.1
<u>Group 5</u>						
SAM1/40B	76.5	26.3	-	-	54.3	20.6
PIP 10	48.9	18.4	-	-	48.5	17.2
7/8/72A	92.8	23.8	-	-	39.1	13.5
7/8/72B	47.1	17.4	-	-	52.6	21.1
6/8/26C	118.1	48.3	-	-	156.9	38.2
6/8/33B	87.8	27.1	-	-	139.3	35.9
6/8/33A	46.8	24.2	-	-	57.5	24.1
<u>Group 6</u>						
6/8/54D	82.6	22.7	-	-	34.7	15.2
6/8/13C	67.5	35.5	-	-	39.6	10.8
6/8/13A	60.9	31.1	-	-	25.8	12.5
6/8/81D	72.8	23.8	-	-	130.4	24.2
PIP 26	61.5	21.7	-	-	90.7	22.1
7/8/21	29.5	12.3	-	-	22.4	11.8
7/8/58B	212.9	68.9	-	-	106.2	28.3

3.2.7. Specificity of monoclonal antibodies for lipopolysaccharide (LPS) from S. minnesota (Wild type) and S. virchow (WT) : Groups 1 to 4.

The LPS-specific Mabs were also assayed with lipopolysaccharide (LPS) preparations from S. minnesota (Wild type) and S. virchow (WT) with the object of determining whether any were cross-reactive with other the LPS of other Salmonella species.

Group 1 (IgA isotype, LPS specific, cross-reactive) Mabs displayed cross-reactivity for S. minnesota (WT) LPS (Table 7). The one Mab in group 2 (IgM isotype, LPS specific, cross-reactive) as in previous assays gave far lower counts than group 1 Mabs but did cross react with S. minnesota (WT) LPS (Table 7). Group 3 (IgA isotype, LPS specific, non cross-reactive) Mabs although specific for S. typhimurium (WT) LPS showed no cross-reaction with S. minnesota (WT) LPS (Table 7). Group 4 (non IgA isotype, LPS specific, non cross-reactive) as with group 3 displayed no cross reactivity with S. minnesota (WT) LPS (Table 7).

None of the Mabs from the four groups showed any cross-reactivity with LPS from S. virchow (WT).

3.2.8. Specificity of monoclonal antibodies for lipopolysaccharide (LPS) from S. minnesota R60 (Ra), R5 (Rc) and R595 (Re) mutants : Groups 1 to 4.

All LPS-specific Mabs were assayed for reactivity with the core region of LPS which is highly conserved between Salmonella species. Mutant strains of S.

minnesota which produce rough LPS devoid of the O antigen side chains have been identified. The actual length of the core structure in these strains differs with some containing the complete region (designated Ra-chemotype). Those with the shortest core are termed Re-chemotype and intermediate in length between Ra and Re are other chemotypes including the Rc chemotype which contains approximately half the core region.

The previous results which indicated cross-reactivity with S. minnesota (WT) LPS for Group 1 and Group 2 Mabs suggested that those which cross-reacted might be specific for the LPS core region. To determine if any of the LPS-specific Mabs were LPS core specific they were assayed for reactivity with LPS core preparations of Ra, Rc and Re chemotypes.

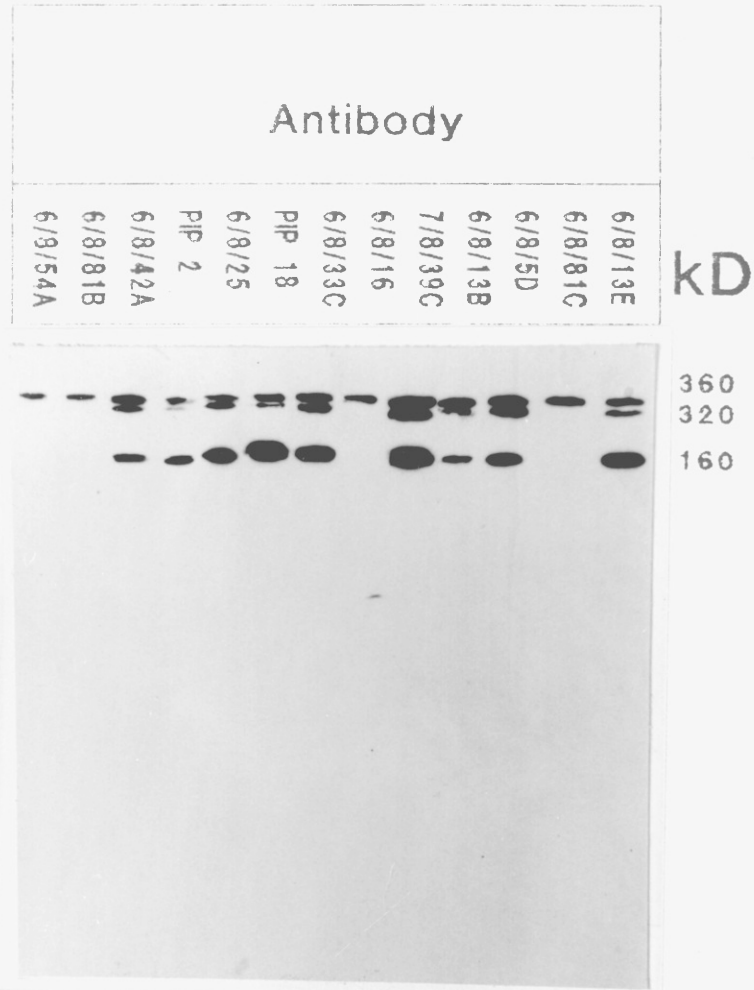
The results (not shown) indicated no specificity by any of the Mabs for any of the preparations. Controls using immune rabbit serum directed against the Re-chemotype reacted with all preparations. Any cross-reactivity by Mabs between S. typhimurium LPS and S. minnesota LPS would not appear to be due to a specificity for the core region of LPS.

3.3 ANALYSIS OF MOLECULAR FORMS OF SELECTED IGA MONOCLONAL ANTIBODIES (MABS).

In order to analyze their molecular forms samples of each of the selected IgA Mabs were run on non-reduced SDS-PAGE gels and probed with radiolabelled antisera specific for rat IgA. The resultant autoradiograph (see Fig 13) shows that many of the IgA Mabs produced up to three molecular forms of 360 kD, 320 kD and 160 kD molecular weight. The others only appeared to produce one molecular form of 360 kD molecular weight.

The 160 kD form would represent the monomeric form of IgA whereas the 360 kD and 320 kD forms are both close to the molecular weight of the dimeric form of IgA. The presence of a dimer at the approximate dimeric molecular weight of IgA could be related to the presence or absence of J-chain, a protein of approximate molecular weight 30 kD which is normally found associated with dimeric IgA. The 360 kD form would represent the dimeric IgA with J-chain. The 320 kD form could result from the loss of J-chain from the 360 kD form by proteolytic digestion or possibly by the lack of addition through some error in the processing of the dimeric IgA by the hybridomas involved.

Figure 13. Autoradiograph of IgA monoclonal antibodies of Groups 1 and 3 separated on by non-reduced SDS-PAGE and probed with Iodine¹²⁵ goat anti-rat IgA antiserum.



3.3.1. Separation of molecular forms of selected IgA monoclonal antibodies PIP2 and PIP18.

PIP18 and PIP2 were chosen as examples of IgA Mabs which produced multiple molecular forms. Each Mab was subjected to the same process of separation to produce preparations of all three molecular forms as described above. Following ammonium sulphate precipitation of protein from maximal growth medium the resuspended IgA was immunoprecipitated, redissolved in a glycine buffer and separated on a Sephacryl column. The absorbance profile for each Mab separation is shown in Figs 14 and 15.

Figure 14. Absorbance profile for the fractions collected by separation of molecular forms of PIP 2 monoclonal antibody on a Sephacryl S-200 column.

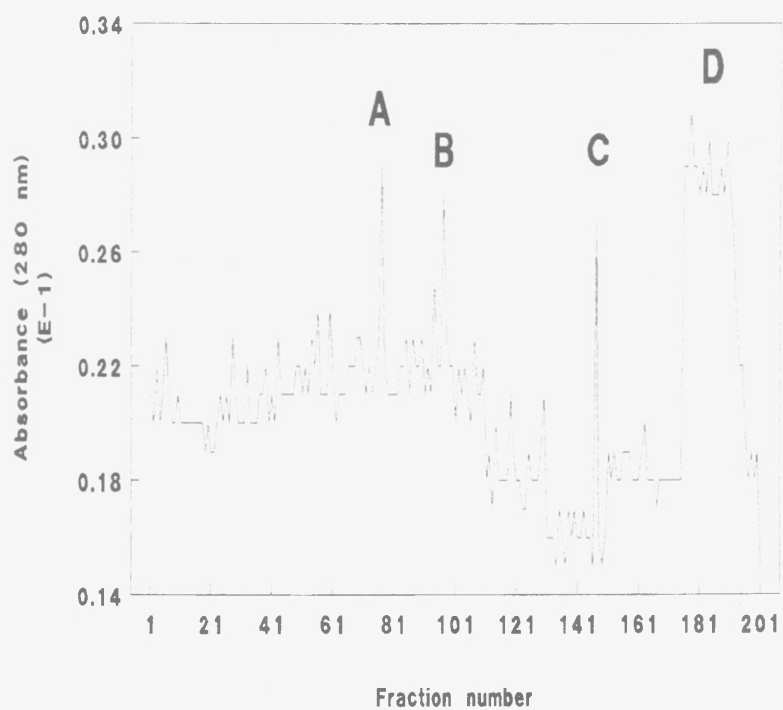
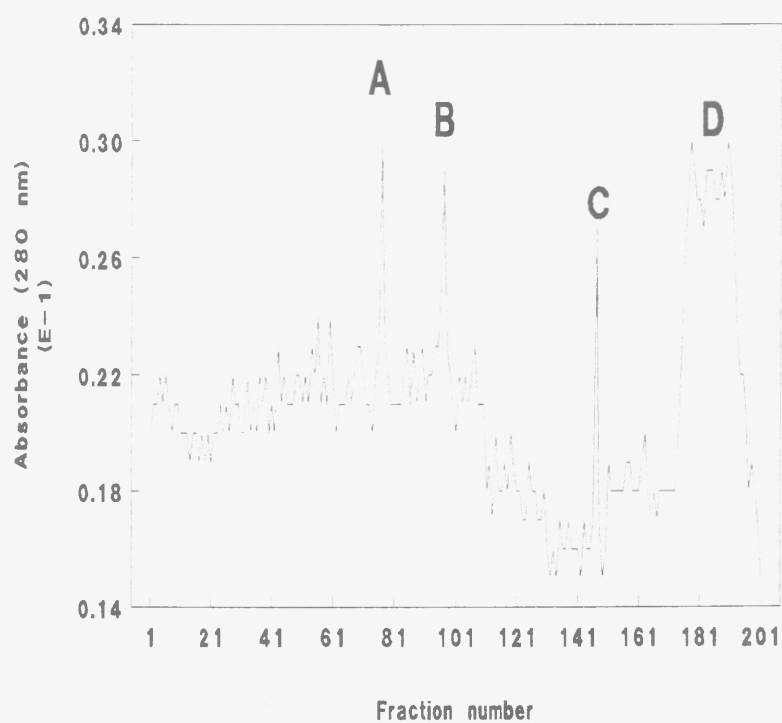


Figure 15. Absorbance profile for the fractions collected by separation of molecular forms of PIP 18 monoclonal antibody on a Sephacryl S-200 column.



The peaks A,B and C correspond to the 360 kD, 320 kD and 160 kD molecular forms of each Mab. The D peak represents the F(ab)₂ preparation and various low molecular weight digested proteins. The fractions of each peak (ie A, B and C) were **pooled** and dialysed to restore the pH to 7.2. Each peak was then run on a non-reduced SDS-PAGE gel to confirm the molecular weight and purity of each molecular form (see Fig 16).

3.4 STANDARDIZATION OF IN VITRO ADHERENCE ASSAY WITH MADIN-DARBY CANINE KIDNEY (MDCK) CELL-LINE FOR ASSESSMENT OF MONOCLONAL ANTIBODIES.

An in vitro adherence and invasion assay was described by Finlay et al. (1988) for S. choleraesuis and Madin Darby canine kidney (MDCK) cell monolayers. This system was also used with S. typhimurium (Finlay, personal communication) however only initial experiments were performed and the results were unpublished. This in vitro assay was adopted for this work to assess the effect of selected monoclonal antibodies on the adherence of S. typhimurium SL/BR to cells in vitro.

3.4.1. Determination of growth conditions for Salmonella typhimurium SL3261 BRD026 (SL/BR) in Eagles minimal essential medium (EMEM).

During the adherence assay the live SL/BR would be capable of replication. It was therefore necessary that the level of SL/BR replication within in EMEM should be

Molecular weights

PIP 2	360kD
PIP 2	320kD
PIP 2	160kD
PIP 18	360kD
PIP 18	320kD
PIP 18	160kD

measured so that the level of adherence after the incubation period could be calculated relative to the final concentration of SL/BR.

Twenty-four well plates containing 2mls of EMEM per well were inoculated with 50 colony forming units of SL/BR from a Nutrient broth culture in log phase. The growth curve for this inocula (see Fig 17) shows an initial lag phase for the first 3 hours after which the level of replication increases so that after 5 hours the culture is in log phase. Log phase continues until 7 hours after which the cultures were terminated.

From these results it was decided that the subsequent adherence assays to assess the effects of the Mabs would be carried out over 3 hours during which time the increase in bacteria within the system would be negligible.

3.4.2. The effect of inoculum size on the adherence of Salmonella typhimurium SL3261 BRD026 (SL/BR) to Madin-Darby canine kidney (MDCK) cell-line.

The effect of initial inoculum size on the time course of adherence of S. typhimurium SL/BR to the MDCK cell monolayers was investigated using inocula ranging from 10^6 to 10^1 colony forming units per well with adherence being measured by both ELISA and colony forming units recovered at hourly intervals over 7 hours.

The results (Fig 18 and 19) show a dose-dependant response with all inocula following the same time course for adherence. All inocula show a lag phase from 0 to 3

Figure 17. Growth curve of S. typhimurium SL3261 BRD026 (SL/BR) in Eagle's modified essential medium (EMEM) at 37°C, 5% carbon dioxide.

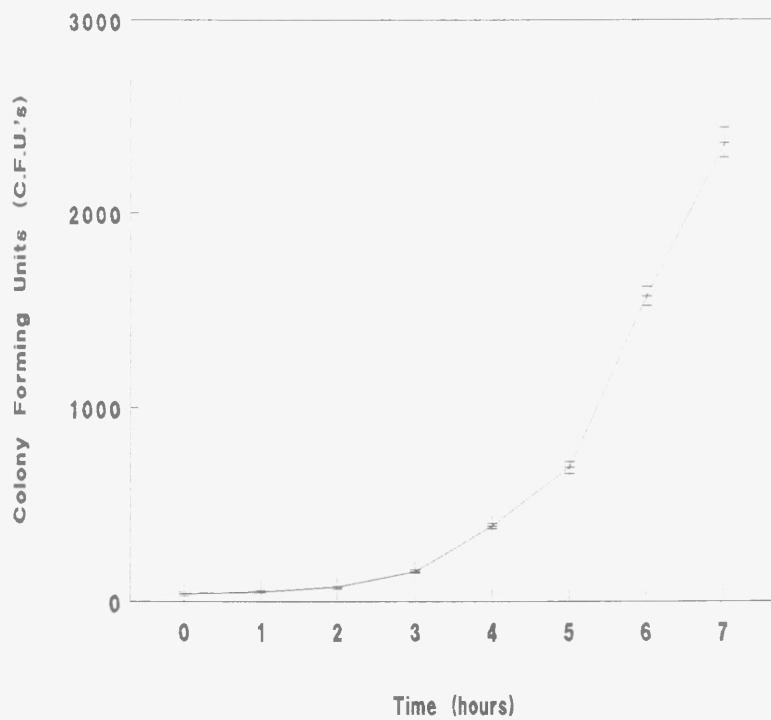


Figure 18. Adherence of S. typhimurium SL3261 BRD026 (SL/BR) to Madin-Darby canine kidney cell (MDCK) monolayers with time as measured by ELISA using varying initial inocula.

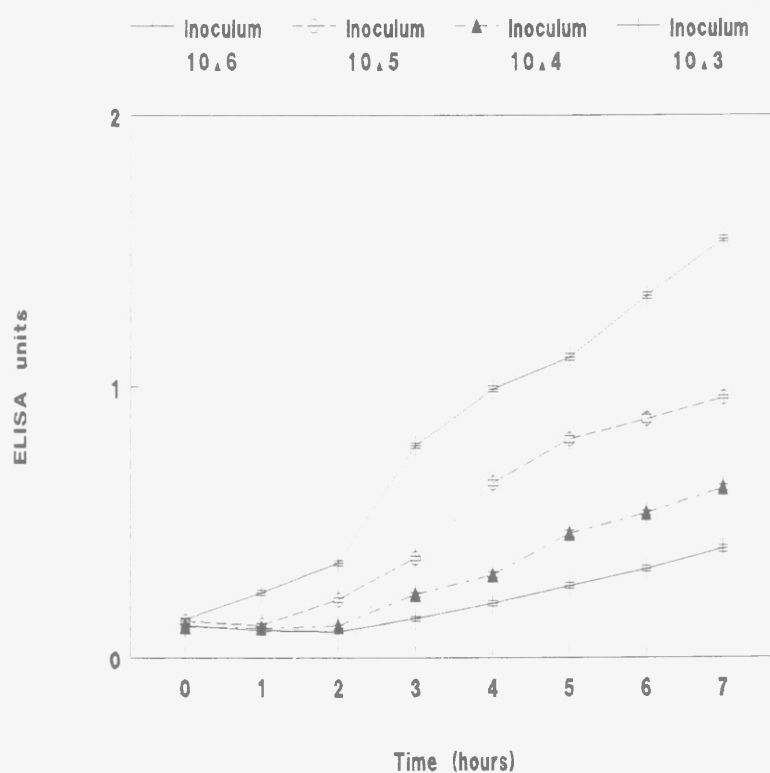
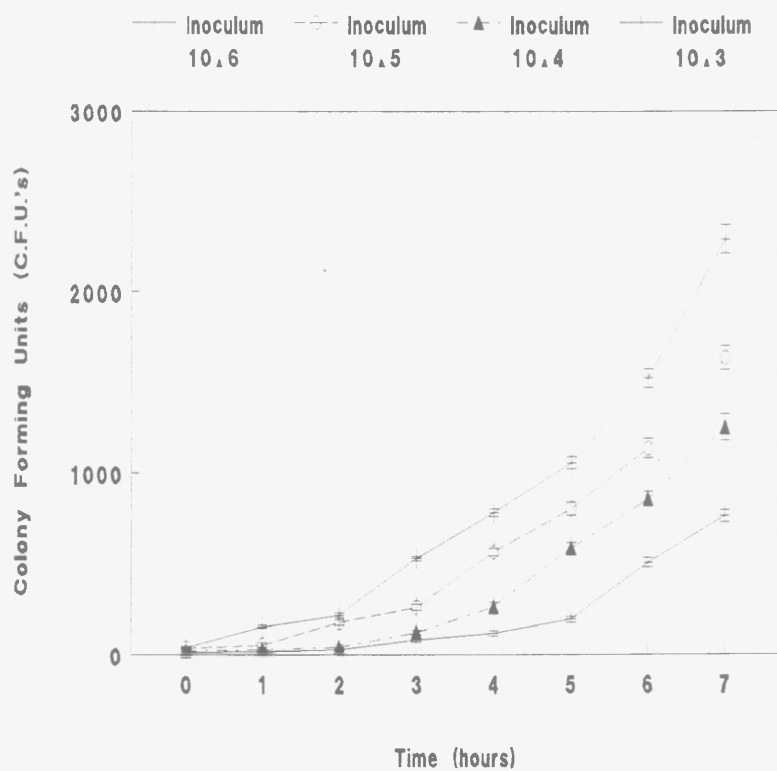


Figure 19. Adherence of S. typhimurium SL3261 BRD026 (SL/BR) to Madin-Darby canine kidney cell (MDCK) monolayers with time as measured by colony forming units (CFU's) using varying initial inocula.



hours after which the rate of increase in adherence becomes exponential. Higher inocula does not overcome the initial lag phase.

Figs 18 and 19 show the same results measured by ELISA and CFU's respectively.

3.4.3. Correlation of ELISA and colony forming units (CFU's) for the assessment of adherence of Salmonella typhimurium SL3261 BRD026 (SL/BR) to Madin-Darby canine kidney (MDCK) cell-line.

The correlation between both the CFU and ELISA measurements of adherence of S. typhimurium SL/BR was assessed for each of the initial inocula sizes and the results are shown in Table 8.

All inocula sizes gave good correlation between both ELISA and CFU measures of adherent bacteria. The best correlation was found with the 10^4 inocula. To allow for efficient processing of assays all further experiments were conducted with only ELISA measures of adherent bacteria. The inoculum size of 10^4 was chosen for further work so that subsequent conversion of ELISA units to actual numbers of viable bacteria (CFU's) could be undertaken with the greatest degree of accuracy.

Table 8. Correlation of measurement of adherence of S. typhimurium SL3261 BRD026 (SL/BR) to Madin-Darby canine kidney cell (MDCK) monolayers using ELISA and colony forming units (CFU's) for various initial inocula.

Inoculum	Gradient	Y-intercept	Correlation coefficient
10 ^{4.5}	0.0014	0.063	0.996
10 ^{4.5}	0.0015	0.074	0.949
10 ^{4.4}	0.0013	0.079	0.987
10 ^{4.3}	0.0006	0.096	0.816
10 ^{4.2}	0.0004	0.101	0.744
10 ^{4.1}	0.0003	0.098	0.707

3.5 FUNCTIONAL ASSESSMENT OF MONOCLONAL ANTIBODIES IN THE PREVENTION OF ADHERENCE OF SALMONELLA TYPHIMURIUM SL3261 BRD026 (SL/BR) TO MADIN-DARBY CANINE KIDNEY (MDCK) CELL-LINE.

As noted above (Section 3.2.1) it could be seen that, although the actual numbers were small, there was a difference between the isotype distribution between those Mabs which were LPS-specific and those which were not. Almost three times as many Mabs specific for LPS were of the IgA isotype as compared with either the IgM or IgG1 isotype Mabs. A principal aim of the project was to investigate the properties of mucosally-derived IgA Mabs as related to mucosal immunity. The primary function of IgA at the mucosal surface is immune exclusion by inhibition of adherence of antigen. The LPS-specific Mabs which contained a high number of IgA isotypes were used in further functional in vitro adherence assays to assess their role and mode of action in inhibiting adherence.

The following results include controls of untreated bacteria for each assay. Further controls of bacteria treated with non-specific rat IgA monoclonal antibody (specific for horse radish peroxidase), non-specific mouse IgM (specific for Neisseria meningitidis type Y capsular polysaccharide) and non-specific mouse IgG1 (specific for Neisseria meningitidis type Y capsular polysaccharide) were included in each assay as well. These controls did not differ significantly from the untreated controls and as such are not quoted.

All assays were carried out using maximal growth medium of each monoclonal antibody.

3.5.1. The effect of monoclonal antibody (Mab) treatment and the treatment with molecular forms of IgA Mabs (PIP2 and PIP18) on the adherence of Salmonella typhimurium SL3261 BRD026 (SL/BR) to Madin-Darby canine kidney (MDCK) cell-line.

An initial assessment was carried out in which both bacteria and monoclonal antibody were added to MDCK cell monolayers. After 3 hours the adherent SL/BR were measured by ELISA.

Group 1 (IgA isotype, LPS specific, cross-reactive) contains 4 Mabs all with similar reactivity for SL/BR in RIA. This was repeated with their inhibition of adherence with an group mean of 27% inhibition compared with controls (Table 9).

Group 2 (IgM isotype, LPS specific, cross-reactive) contains only one Mab which appears to have only low reactivity with SL/BR as compared with Group 1 Mabs but also inhibited adherence at the level of 36% compared to controls (Table 9).

Group 3 (IgA isotype, LPS specific, non cross-reactive) contained 9 Mabs which gave a mean inhibition of adherence of 27% compared with controls (Table 9).

Group 4 (non IgA isotype, LPS specific, non cross-reactive) has four Mabs, 3 of the IgM isotype and 1 IgG1. The 3 IgM Mabs showed inhibition of adherence (28% compared with controls). The IgG1 antibody was the only

Table 9. Adherence of monoclonal antibody treated S. typhimurium SL3261 BRD026 (SL/BR) to Madin-Darby canine kidney cell (MDCK) monolayers measured by both ELISA and colony forming units (CFU's) - Groups 1 to 4 and molecular forms of PIP 2 and PIP 18.

	Initial adherence			
	Colony forming units		ELISA	
		(Sd)		(Sd)
<u>Group 1</u>				
6/8/5D	40	9.5	0.136	0.008
6/8/13E	30	9.9	0.096	0.009
6/8/81C	35	7.2	0.12	0.009
6/8/13B	39	9.4	0.131	0.010
<u>Group 2</u>				
7/8/39C	47	8.8	0.135	0.008
<u>Group 3</u>				
PIP18	41	8.7	0.111	0.009
6/8/54A	53	9.4	0.134	0.010
PIP 2	37	9.5	0.129	0.008
6/8/52C	46	9.7	0.103	0.008
6/8/16	32	7.4	0.128	0.008
6/8/33C	33	9.1	0.129	0.007
6/8/42A	27	9.9	0.111	0.009
6/8/81B	28	7.2	0.108	0.009
6/8/25	47	6.9	0.135	0.009
<u>Group 4</u>				
PIP13	34	8.1	0.109	0.010
7/8/18	42	7.1	0.132	0.006
6/8/53B	34	8.8	0.128	0.008
7/8/64	122	7.1	0.227	0.006
PIP2 (160kD)	-	-	0.113	0.010
PIP2 (320kD)	-	-	0.105	0.008
PIP2 (360kD)	-	-	0.109	0.011
PIP18 (160kD)	-	-	0.112	0.009
PIP18 (320kD)	-	-	0.114	0.006
PIP18 (360kD)	-	-	0.101	0.008
CONTROL	133	8.5	0.221	0.009

Mab tested which gave no inhibition of adherence (97% compared with controls) (Table 9).

Two IgA Mabs which produced multiple molecular forms (PIP18 and PIP2) were purified and the individual molecular forms isolated.

Solutions of 10 ug of protein ml⁻¹ were adjusted for each molecular form and then used in the adherence assay (Table 9).

All forms of both monoclonal antibodies gave rise to inhibition of adherence comparable with that of the unpurified maximal growth medium used for the initial assays described above.

A similar degree of inhibition was shown with each molecular form of each monoclonal antibody. No significant difference was seen between the molecular forms of the same antibody.

3.5.2. The effect of pre-incubation on the effect of monoclonal antibody (Mab) treatment and the adherence of Salmonella typhimurium SL3261 BRD026 (SL/BR) to Madin-Darby canine kidney (MDCK) cell-line.

Initial assays were carried out with both bacteria and monoclonal antibody being added together to the MDCK monolayer. To assess whether pre-incubation of monoclonal antibody and bacteria could further effect adherence a series of experiments were conducted in which bacteria were pre-incubated for varying times from 0 to 60 minutes (Table 10).

Pre-incubation from 0 to 60 minutes had no effect on

Table 10. Effects of pre-incubation on the initial adherence at 0 hours of monoclonal antibody treated S. typhimurium SL3261 BRD026 (SL/BR) to Madin-Darby canine kidney cell (MDCK) monolayers measured by both ELISA and colony forming units (CFU's) - Groups 1 to 4.

	Pre-incubation period (minutes)					
	0	10	20	30	45	60
	(ELISA units)					
<u>Group 1</u>						
6/8/5D	0.118	0.105	0.094	0.077	0.075	0.059
6/8/13E	0.101	0.097	0.086	0.065	0.071	0.068
6/8/81C	0.101	0.090	0.096	0.082	0.075	0.071
6/8/13B	0.105	0.096	0.093	0.078	0.079	0.061
<u>Group 2</u>						
7/8/39C	0.098	0.096	0.088	0.085	0.095	0.089
<u>Group 3</u>						
PIP18	0.112	0.100	0.092	0.078	0.074	0.069
6/8/54A	0.144	0.133	0.133	0.135	0.136	0.125
PIP 2	0.113	0.104	0.094	0.075	0.082	0.067
7/8/52C	0.090	0.087	0.092	0.098	0.103	0.086
6/8/16	0.102	0.096	0.083	0.078	0.068	0.066
6/8/33C	0.113	0.112	0.116	0.099	0.108	0.104
6/8/42A	0.099	0.084	0.083	0.064	0.077	0.061
6/8/81B	0.098	0.092	0.071	0.069	0.074	0.059
6/8/25	0.139	0.129	0.118	0.133	0.133	0.120
<u>Group 4</u>						
PIP 13	0.101	0.091	0.096	0.088	0.094	0.086
7/8/18	0.117	0.110	0.112	0.112	0.108	0.110
7/8/64	0.108	0.111	0.102	0.108	0.099	0.092
6/8/53B	0.108	0.110	0.099	0.097	0.115	0.095
<u>Control</u>	0.117	0.118	0.117	0.120	0.119	0.118

the adherence of antibody treated bacteria as measured after 3 hours.

All antibodies showed an increase in initial inhibition of adherence at time 0 hours when pre-incubated for 60 minutes.

Within group 1 (IgA isotype, LPS specific, cross-reactive) there was a mean increase of inhibition of 38.85% (Table 10), whereas in group 2 (IgM isotype, LPS specific, cross-reactive) the increase was only 9.20% (Table 10).

Group 3 (IgA isotype, LPS specific, non cross-reactive) also gave a large mean increase in initial inhibition (25.77%) (Table 10).

Group 4 (non IgA isotype, LPS specific, non cross-reactive) gave a much lower mean increase of only 9.95% (Table 10).

3.5.3. The effect of the time of addition of monoclonal antibody (Mab) on the adherence of Salmonella typhimurium SL3261 BRD026 (SL/BR) to Madin-Darby canine kidney (MDCK) cell-line.

The adherence assays were carried out over a period of 3 hours during which the SL/BR adhered to the MDCK monolayer. In an attempt to discover whether it was necessary for the antibody to be present from the start of the assay a series of experiments were performed to assess the effect of adding the bacteria at 0 hours and the monoclonal antibody at times between 0 and 3 hours.

Table 11. Effects of post-incubation on the adherence after 3 hours of monoclonal antibody treated S. typhimurium SL3261 BRD026 (SL/BR) to Madin-Darby canine kidney cell (MDCK) monolayers measured by both ELISA and colony forming units (CFU's) - Groups 1 to 4.

	Time of addition of Mab (Hours)							
	0	(Sd)	1	(Sd)	2	(Sd)	3	(Sd)
	(ELISA units)							
<u>Group 1</u>								
6/8/5D	0.129	0.016	0.139	0.019	0.152	0.016	0.183	0.017
6/8/13E	0.118	0.017	0.127	0.018	0.164	0.018	0.184	0.018
6/8/81C	0.125	0.019	0.125	0.019	0.153	0.020	0.211	0.017
6/8/13B	0.129	0.018	0.140	0.017	0.166	0.015	0.210	0.016
<u>Group 2</u>								
7/8/39C	0.135	0.019	0.137	0.015	0.165	0.018	0.193	0.016
<u>Group 3</u>								
PIP18	0.136	0.016	0.146	0.018	0.173	0.017	0.196	0.017
6/8/54A	0.133	0.017	0.148	0.018	0.173	0.018	0.219	0.014
PIP 2	0.132	0.016	0.131	0.016	0.149	0.014	0.189	0.019
7/8/52C	0.110	0.017	0.105	0.017	0.159	0.017	0.202	0.015
6/8/16	0.130	0.016	0.132	0.017	0.159	0.017	0.204	0.017
6/8/33C	0.124	0.014	0.128	0.017	0.152	0.018	0.193	0.014
6/8/42A	0.112	0.016	0.123	0.014	0.161	0.018	0.22	0.017
6/8/81B	0.107	0.019	0.108	0.017	0.151	0.017	0.187	0.013
6/8/25	0.128	0.018	0.144	0.014	0.158	0.018	0.195	0.017
<u>Group 4</u>								
PIP 13	0.120	0.017	0.145	0.019	0.166	0.019	0.188	0.015
7/8/18	0.122	0.017	0.142	0.017	0.165	0.017	0.196	0.020
7/8/64	0.228	0.017	0.226	0.018	0.214	0.016	0.221	0.018
6/8/53B	0.135	0.017	0.145	0.019	0.160	0.019	0.186	0.013
<u>Control</u>	0.232	0.017	0.229	0.018	0.234	0.020	0.230	0.016

The results are shown in Table 11. For all antibodies addition after 2 hours gave only slight reductions in adherence compared with controls. It was necessary to add the antibody between 0 and 1 hours to produce any inhibition with greatest inhibition occurring with addition at the start of the assay (0 hours).

3.5.4. The effect of addition of monoclonal antibody (Mab) on Salmonella typhimurium SL3261 BRD026 (SL/BR) adherent to Madin-Darby canine kidney (MDCK) cell-line.

To discover whether antibodies had any effect on bacteria which had already adhered to the MDCK monolayer a series of experiments were carried out in which monolayers were incubated for 3 hours with SL/BR after which they were washed and monoclonal antibodies were then added. The monolayers were then re-incubated for a further 3 hours and the level of adherence measured and compared to controls to which no antibody was added.

As can be seen from the results (Table 12) the monoclonal antibodies had no effect on the bacteria already adherent to the MDCK monolayer. The antibodies did not reduce the level of adherence after addition and the level of adherence increased over the 3 hours in parallel with the controls. However a reduction occurred over the 3 hours with added antibody such that at 6 hours there was a reduction in adherence compared with controls.

Table 12. Effect of monoclonal antibody addition on S. typhimurium SL3261 BRD026 (SL/BR) adherent to Madin-Darby canine kidney cell (MDCK) monolayers measured by both ELISA and colony forming units (CFU's) - Groups 1 to 4.

	Time (Hours)							
	0	(Sd)	1	(Sd)	2	(Sd)	3	(Sd)
	(ELISA units)							
<u>Group 1</u>								
6/8/5D	0.201	0.017	0.253	0.019	0.288	0.018	0.318	0.019
6/8/13E	0.227	0.018	0.254	0.013	0.298	0.018	0.327	0.016
6/8/81C	0.217	0.017	0.245	0.017	0.287	0.016	0.313	0.014
6/8/13B	0.224	0.017	0.252	0.019	0.287	0.016	0.311	0.015
<u>Group 2</u>								
7/8/39C	0.232	0.014	0.276	0.016	0.333	0.017	0.381	0.014
<u>Group 3</u>								
PIP 18	0.223	0.018	0.247	0.021	0.287	0.017	0.314	0.017
6/8/54A	0.224	0.019	0.276	0.017	0.325	0.021	0.375	0.019
PIP 2	0.198	0.014	0.248	0.018	0.278	0.016	0.305	0.017
7/8/52C	0.216	0.014	0.278	0.015	0.337	0.019	0.371	0.014
6/8/16	0.211	0.016	0.255	0.017	0.301	0.017	0.320	0.014
6/8/33C	0.216	0.019	0.257	0.016	0.309	0.019	0.350	0.015
6/8/42A	0.227	0.020	0.249	0.016	0.304	0.016	0.334	0.017
6/8/81B	0.212	0.019	0.253	0.017	0.283	0.020	0.312	0.017
6/8/25	0.218	0.017	0.250	0.016	0.294	0.017	0.354	0.015
<u>Group 4</u>								
PIP 13	0.213	0.015	0.256	0.015	0.289	0.016	0.311	0.018
7/8/18	0.221	0.014	0.247	0.016	0.278	0.018	0.310	0.018
7/8/64	0.225	0.022	0.279	0.016	0.327	0.019	0.379	0.017
6/8/53B	0.214	0.015	0.247	0.014	0.287	0.016	0.313	0.019
CONTROL	0.221	0.016	0.281	0.015	0.341	0.018	0.403	0.016

3.5.5. The effect of competition of monoclonal antibody (Mab) of differing isotypes on Salmonella typhimurium SL3261 BRD026 (SL/BR) adherence to Madin-Darby canine kidney (MDCK) cell-line.

The IgG1 antibody (7/8/64) appeared to be the only antibody which did not inhibit adherence of SL/BR to MDCK cell monolayers. Radioimmunoassay data showed that 7/8/64 significantly bound SL/BR. As it seemed reasonable that 7/8/64 was binding SL/BR in the MDCK cell adherence assay a series of experiments were carried out to investigate whether it would actively compete with the other antibodies and reduce the inhibitory effect.

Two IgA antibodies (6/8/5D and PIP2) from group 1 and two IgM antibodies (7/8/39C and PIP13) from group 2 and 4 were titrated individually in competition with 7/8/64. Equal volumes of the two antibodies were added in each assay. The level of IgG1 antibody was held at the ABT50% value whilst the other antibody was titrated over a number of wells from 4x TI^{50%} to 0.125x TI^{50%}. The arrangements were then reversed and the inhibitory antibodies were added at the TI^{50%} concentration with the IgG1 antibody titrated from 4x ABT^{50%} to 0.125x ABT^{50%}.

Titration of the IgG1 antibody against both IgA and IgM antibodies were similar (Figs 20 to 23). A reduction was seen in the inhibitory effect of the IgA and IgM antibodies when titrated with IgG1 at a fixed concentration. Conversely when titrating IgG1 with the IgA and IgM antibodies there

Figure 20. Inhibition of adherence monoclonal antibody treated S. typhimurium SL3261 BRD026 (SL/BR) to Madin-Darby canine kidney cell (MDCK) monolayers measured by ELISA - Competition assay between 6/8/5D (IgA, Group1) and 7/8/64 (IgG1, Group 4).

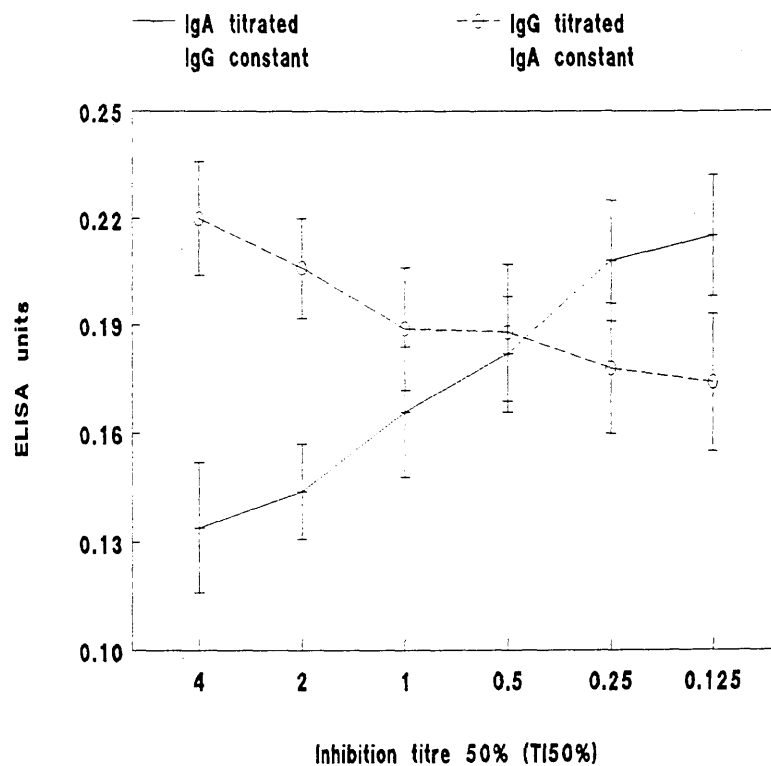


Figure 21. Inhibition of adherence monoclonal antibody treated S. typhimurium SL3261 BRD026 (SL/BR) to Madin-Darby canine kidney cell (MDCK) monolayers measured by ELISA - Competition assay between 7/8/39C(IgM, Group2) and 7/8/64 (IgG1, Group 4).

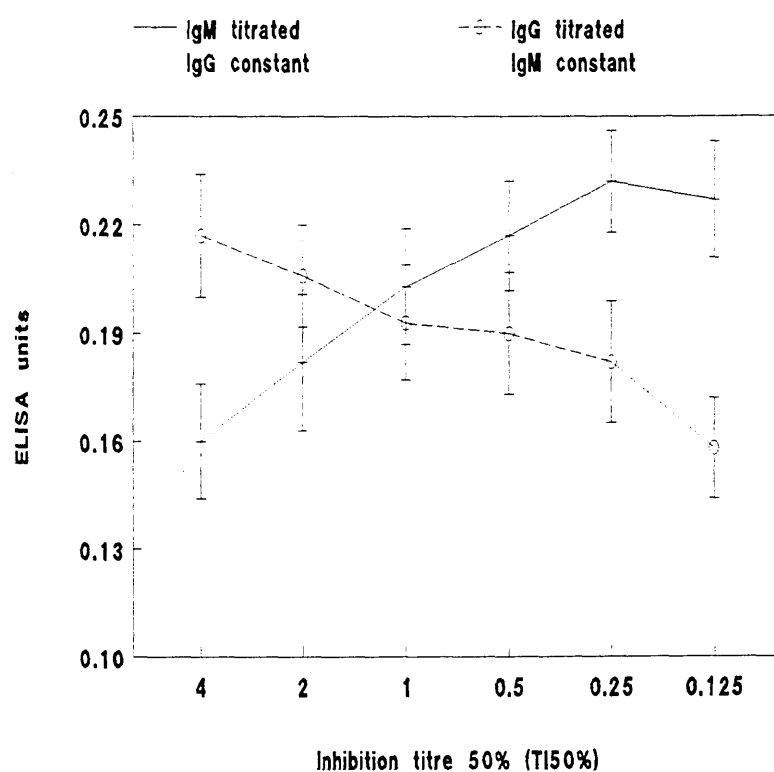


Figure 22. Inhibition of adherence monoclonal antibody treated S. typhimurium SL3261 BRD026 (SL/BR) to Madin-Darby canine kidney cell (MDCK) monolayers measured by ELISA - Competition assay between PIP 2 (IgA, Group3) and 7/8/64 (IgG1, Group 4).

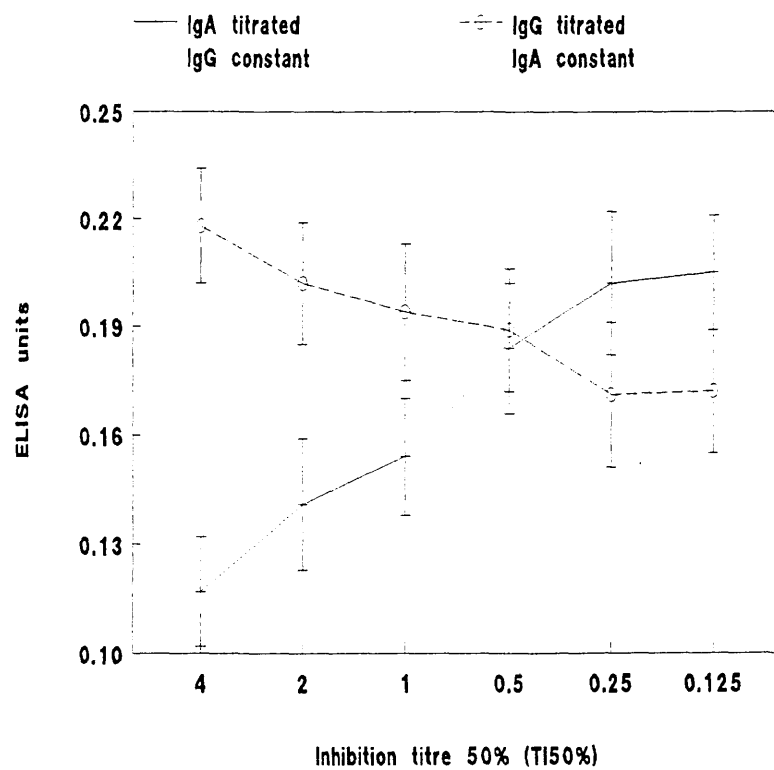
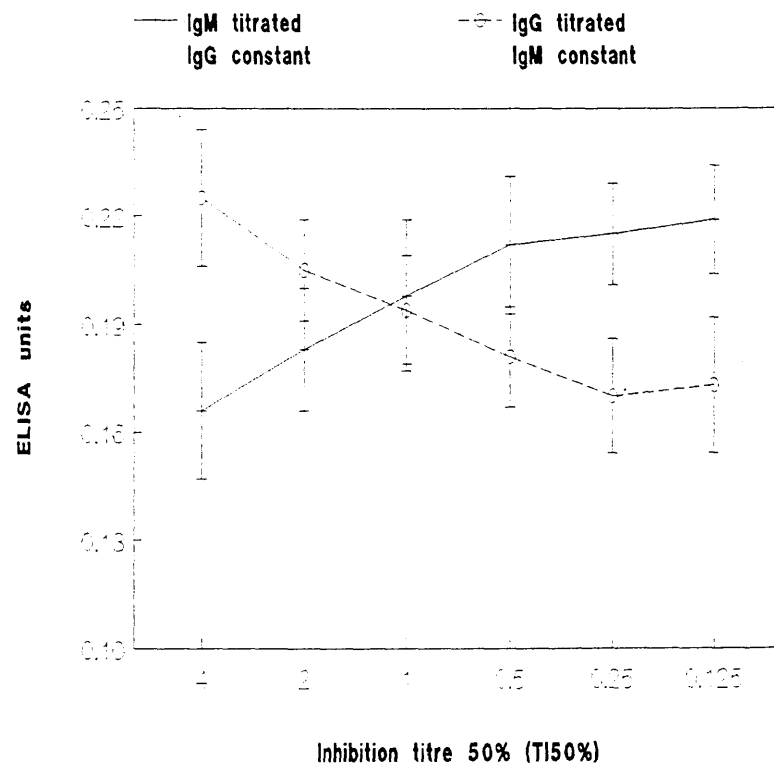


Figure 23. Inhibition of adherence monoclonal antibody treated S. typhimurium SL3261 BRD026 (SL/BR) to Madin-Darby canine kidney cell (MDCK) monolayers measured by ELISA - Competition assay between PIP 13 (IgM, Group4) and 7/8/64 (IgG1, Group 4).



was an increase in the inhibition of adherence as the IgG1 concentration declined.

The results indicate that the IgG1 antibody has an active effect on the adherence of SL/BR to MDCK cells such that at high concentrations it will reduce the inhibitory effect seen with the IgA and IgM antibodies tested.

Also, plotting the titration curves of adherence versus inhibitory concentration of each pair (Figs 20 to 23) revealed that for the IgA antibodies the cross-over point of the curves occurred at approximately $0.5 \times \text{TI}^{50\%}$, whilst the IgM antibodies had a cross-over point of approximately $1.0 \times \text{TI}^{50\%}$.

3.6 THE EFFECT OF NON-SPECIFIC FACTORS ON THE ADHERENCE OF SALMONELLA TYPHIMURIUM SL3261 BRD026 (SL/BR) TO MADIN-DARBY CANINE KIDNEY (MDCK) CELL-LINE.

The initial adherence of S. typhimurium to MDCK cells occurred primarily during the first 3 hours. Preliminary experiments were carried out to assess whether this adherence was an intrinsically specific or non-specific function.

The presence of a specific receptor-adhesin system utilizing lipopolysaccharide (LPS) was investigated. If present the action of the selected LPS-specific monoclonal antibodies could then be further assessed by comparison with the non-LPS specific monoclonal antibodies.

MDCK cell monolayers produce a high negative charge on their apical surface. Finlay et al. (1988) found that S. choleraesuis would only adhere to the apical surface in significant numbers. Reducing the apical negative surface charge of MDCK cell monolayers by the addition of Amiloride (a drug which acts by inhibiting sodium transport but has no effect on the viability of MDCK cells) was investigated to assess whether the adherence was influenced by non-specific factors.

3.6.1. Effect of free S. typhimurium (WT) lipopolysaccharide (LPS) on the adherence of Salmonella typhimurium SL3261 BRD026 (SL/BR) to Madin-Darby canine kidney (MDCK) cell-line.

Lipopolysaccharide (LPS) has been shown to be associated with adherence of S. typhimurium to cells both in vitro and in vivo (Kihlstrom, 1977, Perers, 1977). Direct evidence of LPS acting as an adhesin specific for an epithelial cell surface receptor molecule has not been found. Studies using receptor analogues or isolated adhesins have been carried out for a variety of adherent enterobacteriaceae (Smyth et al., 1978, Watt and Ward, 1980)). Free LPS was added to the adherence assay at various concentrations to assess whether it would act as a putative isolated adhesin by inhibiting adherence in a dose-dependant fashion.

No significant inhibition of adherence was seen with any concentration of LPS indicating that it did not compete with the adherence of S. typhimurium SL/BR (Table

13). There did appear to be an increase in adherence compared with controls with increasing concentrations of LPS, although this was not statistically significant.

3.6.2. Effect of Amiloride on the adherence of monoclonal antibody treated and untreated Salmonella typhimurium SL3261 BRD026 (SL/BR) to Madin-Darby canine kidney (MDCK) cell-line.

Bentley (1968) described the effect of Amiloride a potent inhibitor of sodium transport on intact epithelia in vitro. Amiloride has been shown to reduce the electrical potential produced by intact MDCK cell monolayers without affecting cell viability (Cereijido et al., 1978).

Experiments with varying concentrations of Amiloride were carried out to assess whether the reduction of the negative charge on the apical surface of MDCK cell monolayers would result in an increase or decrease in adherence of S. typhimurium (SL/BR) in the presence or absence of monoclonal antibodies.

The concentrations used ranged from that which had been shown to have no effect on the electrical potential of the MDCK monolayers (10^{-8} M) to that which had been shown to destroy the electrical potential entirely (10^{-5} M) (Cereijido et al., 1978). The inhibition of adherence correlated with the reduction of the electrical potential and so the negative charge on the apical surface of the monolayers.

As can be seen from the results (Table 14) the

Table 13. Effect of free S. typhimurium (WT) lipopolysaccharide (LPS) on the adherence of S. typhimurium SL3261 BRD026 (SL/BR) to Madin-Darby canine kidney cell (MDCK) monolayers measured by ELISA.

<u>S. typhimurium</u> (WT) LPS (ug ml ⁻¹)	Adherence (ELISA units) (Sd)	
0 (Control)	0.234	0.018
1	0.235	0.020
2	0.233	0.015
5	0.241	0.014
10	0.238	0.017
15	0.243	0.016
20	0.248	0.017

Table 14. Effect of Amiloride on the adherence of monoclonal antibody treated and untreated S. typhimurium SL3261 BRD026 (SL/BR) to Madin-Darby canine kidney cell (MDCK) monolayers after 3 hours as measured by ELISA - Groups 1 to 4.

	Amiloride concentration (M)					
	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	None
	(ELISA units)					
<u>Group 1</u>						
6/8/5D	0.155	0.155	0.151	0.139	0.136	0.131
6/8/13E	0.161	0.156	0.149	0.136	0.116	0.122
6/8/81C	0.146	0.145	0.133	0.123	0.121	0.122
6/8/13B	0.159	0.158	0.145	0.139	0.129	0.127
<u>Group 2</u>						
7/8/39C	0.175	0.161	0.154	0.142	0.137	0.127
<u>Group 3</u>						
PIP18	0.162	0.157	0.143	0.13	0.131	0.135
6/54A	0.171	0.168	0.15	0.147	0.137	0.136
PIP 2	0.163	0.159	0.145	0.133	0.130	0.121
7/52C	0.163	0.167	0.148	0.132	0.105	0.109
6/8/16	0.151	0.150	0.149	0.141	0.129	0.124
6/33C	0.155	0.147	0.142	0.135	0.131	0.131
6/42A	0.169	0.148	0.143	0.129	0.116	0.108
6/81B	0.163	0.163	0.150	0.135	0.108	0.109
6/8/25	0.159	0.152	0.146	0.141	0.140	0.131
<u>Group 4</u>						
PIP 13	0.156	0.149	0.144	0.127	0.114	0.117
7/8/18	0.157	0.151	0.140	0.132	0.134	0.125
7/8/64	0.203	0.202	0.208	0.217	0.231	0.229
6/8/53B	0.153	0.145	0.141	0.133	0.125	0.132
Control	0.191	0.205	0.206	0.206	0.217	0.229

adherence of S. typhimurium (SL/BR) to MDCK cell monolayers was not inhibited by increasing concentrations of Amiloride.

But Amiloride was seen to ^{inhibit} the adherence of monoclonal antibody treated SL/BR. The results (Table 14) show that with increasing Amiloride concentration the inhibitory effect of the IgA and IgM antibodies of Groups 1 to 4 are reduced in a dose dependant fashion. However the IgG1 antibody (7/8/64) treated SL/BR appears to act in the same way as the untreated bacteria giving a reduction in adherence with increasing Amiloride concentration (Table 14).

3.7 PHYSICO-CHEMICAL ANALYSIS OF MONOCLONAL ANTIBODY (MAB) TREATED SALMONELLA TYPHIMURIUM SL3261 BRD026 (SL/BR).

The adherence of bacteria to mammalian cells has been shown to be influenced not only by the presence of specific receptor mechanisms but also by non-specific physico-chemical interactions. Hydrophobicity and charge on the surface of bacteria have been shown to effect their adherence to mammalian cells in various in vitro assays.

The physicochemical state of SL/BR treated with the Mabs, used in the adherence assay described above, was investigated in an attempt to correlate the effect on adherence to either a change in hydrophobicity or surface charge.

3.7.1. Assessment of bacterial surface hydrophobicity of monoclonal antibody (Mab) treated Salmonella typhimurium SL3261 BRD026 (SL/BR) using Octan-1-ol phase separation.

Assessment of hydrophobicity by partition between an aqueous phase and an octan-1-ol phase was used to determine if treatment of SL/BR with Mabs (Group 1 to 4) had any effect. Untreated SL/BR was used as a control in all assays and was found to be hydrophilic with a hydrophobicity percentage of 36% (sd: 3.4%).

Group 1 (IgA isotype, LPS specific, cross-reactive) contained 4 Mabs all with similar reactivity for SL/BR. Each of the Mabs reduced the hydrophobicity of the SL/BR (mean: 25%, sd: 7.4%) (Table 15).

Group 2 (IgM isotype, LPS specific, cross-reactive) contains only one Mab which appears to have only low reactivity with SL/BR as compared with Group 1 Mabs. Treatment with the Mab had no effect on the hydrophobicity of SL/BR (33%, sd: 5.5%) (Table 15).

Group 3 (IgA isotype, LPS specific, non cross-reactive) contains 9 Mabs which exhibited a range of reactivities with SL/BR. As with Group 1 all Mabs within this group produced a reduction in the hydrophobicity of treated SL/BR (mean: 22%, sd: 5.0%) (Table 15).

Group 4 (non IgA isotype, LPS specific, non cross-reactive) has four Mabs, 3 of the IgM isotype and 1 IgG1. No significant difference is seen in their reactivity with SL/BR. Within this group there was a distinct difference in

Table 15. Hydrophobicity of monoclonal antibody treated S. typhimurium SL3261 BRD026 (SL/BR) to Madin-Darby canine kidney cell (MDCK) monolayers as measured by Octan-1-ol partitioning and hydrophobic interaction chromatography (HIC) - Groups 1 to 4 and molecular forms of PIP 2 and PIP 18.

	Hydrophobicity (%)			
	Octan-1-ol	partitioning	HIC	
	(Sd)		(Sd)	
<u>Group 1</u>				
6/8/5D	17	9.8	18	1.9
6/8/13E	24	8.9	21	3.2
6/8/81C	24	2.4	22	1.7
6/8/13B	35	6.1	26	2.4
<u>Group 2</u>				
7/8/39C	33	2.1	33	1.4
<u>Group 3</u>				
PIP 18	20	9.2	18	2.8
6/8/54A	25	5.6	25	1.2
PIP 2	20	6.4	21	1.7
7/8/52C	29	4.8	29	3.1
6/8/16	19	5.7	15	2.5
6/8/33C	18	7.5	17	2.1
6/8/42A	18	2.6	17	1.6
6/8/81B	18	7.6	18	1.9
6/8/25	31	5.3	24	2.4
<u>Group 4</u>				
PIP 13	39	8.3	36	3.4
7/8/18	38	5.2	34	1.9
6/8/53B	49	5.7	42	3.1
7/8/64	64	6.5	66	1.8
PIP2 (160kD)	-	-	21	1.7
PIP2 (320kD)	-	-	19	2.1
PIP2 (360kD)	-	-	18	1.8
PIP18 (160kD)	-	-	19	3.1
PIP18 (320kD)	-	-	21	2.6
PIP18 (360kD)	-	-	16	2.5
CONTROL	36	3.4	35	2.1

the effect on hydrophobicity of treated SL/BR. The three IgM Mabs had no effect on the hydrophobicity of the treated SL/BR (mean: 42%, sd: 6.1%) whilst the IgG1 antibody significantly increased the hydrophobicity (64%, sd: 6.5%) (Table 15).

These results were subject to large variation between assays as seen by the standard deviation of the results of individual monoclonal antibodies. In order to confirm their initial assessment of the hydrophobicity a further assay system was employed using hydrophobic interaction chromatography (HIC).

3.7.2. Assessment of bacterial surface hydrophobicity of monoclonal antibody (Mab) treated Salmonella typhimurium SL3261 BRD026 (SL/BR) using hydrophobic interaction chromatography (HIC).

Hydrophobic interaction chromatography (HIC), as a method of assessing hydrophobicity, was used to confirm the results obtained with octan-1-ol:aqueous partitioning. The results were expressed as the percentage of bacteria adherent to a column of octyl sepharose after elution with a 0.01 M phosphate buffer pH 6.8 containing 0.1 % (v/v) Triton X-100 buffer. It was hoped that HIC would provide greater accuracy in determining whether treatment of SL/BR with Mabs (Group 1 to 4) had an effect on the hydrophobicity of the antibody bound bacteria compared with controls. Untreated SL/BR was used as a control in all assays and was found to be

hydrophilic with a hydrophobicity index of 35% (sd: 2.1%).

Group 1 (IgA isotype, LPS specific, cross-reactive) contained 4 Mabs all with similar reactivity for SL/BR. Each of the Mabs reduced the hydrophobicity of the SL/BR (mean: 22%, sd: 3.3) (Table 15).

Group 2 (IgM isotype, LPS specific, cross-reactive) contains only one Mab which appears to have only low reactivity with SL/BR as compared with Group 1 Mabs. Treatment with this Mab had no effect on the hydrophobicity of SL/BR as measured by this assay (33%, sd: 3.5%) (Table 15).

Group 3 (IgA isotype, LPS specific, non cross-reactive) contains 9 Mabs which exhibit a range of reactivities with SL/BR. As with Group 1 all Mabs within this group produced a reduction in the hydrophobicity of treated SL/BR (mean: 20%, sd: 4.6%) (Table 15). Group 4 (non IgA isotype, LPS specific, non cross-reactive) has four Mabs, 3 of the IgM isotype and 1 IgG1. No significant difference was seen in their reactivity with SL/BR. Within this group there was a distinct difference in the effect on hydrophobicity of treated SL/BR. The three IgM Mabs had no effect on the hydrophobicity of the treated SL/BR (mean: 37%, sd: 4.1%) whilst the IgG1 antibody significantly increased the hydrophobicity (66%, sd: 1.8%) (Table 15).

These results showed little variation as seen by the standard deviation for each individual monoclonal antibody. They confirmed the initial assessment by octan-

1-ol:aqueous partitioning of the hydrophobicity of treated SL/BR, with significant correlation between results produced by the two methods.

3.7.3. Assessment of bacterial surface hydrophobicity of Salmonella typhimurium SL3261 BRD026 (SL/BR) treated with molecular forms of IgA monoclonal antibody (Mab) using hydrophobic interaction chromatography (HIC): (PIP2 and PIP18).

The molecular forms of two IgA Mabs (PIP18 and PIP2) were assessed for their effects on the hydrophobicity of treated SL/BR.

The hydrophobicity was measured only by hydrophobic interaction chromatography (HIC).

All forms of both monoclonal antibodies caused a decrease in the hydrophobicity of SL/BR comparable with that of the unpurified maximal growth medium used for the initial assays described above (Table 15).

A similar degree of decrease in hydrophobicity was shown with each molecular form of each monoclonal antibody. No significant difference was seen between the decrease in hydrophobicity caused by the molecular forms of the same antibody.

3.7.4. Assessment of bacterial net surface charge of monoclonal antibody (Mab) treated Salmonella typhimurium SL3261 BRD026 (SL/BR) using aqueous two-phase polymer systems.

Bacterial net surface charge of monoclonal antibody (Mab) treated SL/BR was determined by partitioning in two

aqueous two-phase polymer systems. The two systems comprised a non-charged system with sodium chloride as an equally partitioning inorganic salt and a charged system with unequal partitioning of sodium sulphate creating a negatively charged bottom-phase. By comparing the partition coefficients of treated and untreated bacteria in the two systems the relative net bacterial surface charge can be estimated.

Relative net surface charge was calculated from the difference in the partition coefficients of the two systems using the equation:

$$\ln K_p = \ln K^0_p + \frac{FZ}{RT} (U_2 - U_1)$$

where K_p is the partition coefficient in a charged system, K^0_p is the partition coefficient in a non-charged system and Z is the surface charge on the bacteria. R , T , F and $(U_2 - U_1)$ are all constants.

From this equation it can be deduced that the relative surface charge on the bacteria equals:

$$C Z = \ln K_p - \ln K^0_p$$

where C is a composite constant assumed to be 1.

Using this method group 1 (IgA isotype, LPS specific, cross-reactive) monoclonal antibodies are shown to have a mean negative surface charge of 0.49 (sd: 0.12) (Table 16).

Table 16. Surface charge characteristics of monoclonal antibody treated and untreated S. typhimurium SL3261 BRD026 (SL/BR) to Madin-Darby canine kidney cell (MDCK) monolayers as measured by aqueous two-phase polymer partitioning with varying buffer systems and under varying pH - Groups 1 to 4 and molecular forms of PIP 2 and PIP 18.

	Surface charge	
	Relative negative surface charge	Pseudo-isoelectric point
Group 1		
6/8/5D	0.63	6.2
6/8/13E	0.39	5.5
6/8/81C	0.54	6.0
6/8/13B	0.39	6.3
Group 2		
7/8/39C	0.65	6.5
Group 3		
PIP 18	0.45	5.7
6/8/54A	1.03	5.8
PIP 2	0.54	6.2
6/8/52C	0.16	5.8
6/8/16	0.74	5.9
6/8/33C	0.89	5.8
6/8/42A	1.13	5.2
6/8/81B	0.63	5.5
6/8/25	0.21	6.4
Group 4		
PIP 13	0.31	6.1
7/8/18	0.34	6.7
6/8/53B	0.64	6.6
7/8/64	0.00	7.3
PIP2 (160kD)	0.00	-
PIP2 (320kD)	0.21	-
PIP2 (360kD)	0.60	-
PIP18 (160kD)	0.00	-
PIP18 (320kD)	0.32	-
PIP18 (360kD)	0.50	-
CONTROL	0.04	6.9

Group 2 (IgM isotype, LPS specific, cross-reactive) contains only one Mab which gives a mean negative surface charge of 0.65 (Table 16).

Group 3 (IgA isotype, LPS specific, non cross-reactive) contains 9 Mabs all of which increase the net negative surface charge of treated SL/BR (mean: 0.64, sd: 0.34). Group 4 (non IgA isotype, LPS specific, non cross-reactive) has four Mabs, 3 of the IgM isotype and 1 IgG1. No significant difference is seen in the negative surface charge caused by the 3 IgM antibodies (mean: 0.43, sd: 0.18). However the IgG1 antibody does not effect the surface charge of the bacteria (0.0) (Table 16).

3.7.5. Assessment of bacterial net surface charge of Salmonella typhimurium SL3261 BRD026 (SL/BR) treated with molecular forms of IgA monoclonal antibody (Mab) using aqueous two-phase polymer systems (PIP2 and PIP18).

The molecular forms of two IgA Mabs (PIP18 and PIP2) were assessed for their effects on the surface charge of treated SL/BR.

The relative surface charge was measured using aqueous two phase polymer system partitioning and calculated as above.

Both 360 kD forms of the two monoclonal antibodies caused an increase in the net negative surface charge of SL/BR comparable with that of the unpurified maximal growth medium used for the initial assays described above. The 330 kD forms of the two monoclonal antibodies

also increased the net negative surface charge but to a lesser extent (Table 16).

A similar degree of increase in the net negative surface charge was not seen with either 160 kD molecular form of the two monoclonal antibodies. No significant increase was seen in the negative surface charge of SL/BR treated with either of the 160 kD molecular forms of either antibody.

3.7.6. Assessment of pseudo-isoelectric point based on bacterial net surface charge of monoclonal antibody (Mab) treated Salmonella typhimurium SL3261 BRD026 (SL/BR) using aqueous two-phase polymer systems.

The isoelectric point of a protein is that pH at which the total charge on the protein is zero. A pseudo-isoelectric point of monoclonal antibody (Mab) treated SL/BR was determined by partitioning in aqueous two-phase polymer systems under a variety of pH conditions (Albertsson, 1987). The two systems, charged and non-charged as above, were used to assess the partition coefficients of Mab treated bacteria at varying pH. Plotting the partition coefficient against pH for the two systems resulted in two curves the intersection of which represented the pseudo-isoelectric point for each Mab treated bacteria. The pseudo-isoelectric point was used as a measure of the surface charge of the Mab treated bacteria.

Group 1 (IgA isotype, LPS specific, cross-reactive) contains 4 Mabs. The pseudo-isoelectric point of treated

SL/BR was reduced by all of the antibodies (mean: pH 6.0, sd:0.36) (Table 16).

Group 2 (IgM isotype, LPS specific, cross-reactive) contains only one Mab which although it had only low reactivity with SL/BR compared with group 1 Mabs gave a reduction of the pseudo-isoelectric point of treated SL/BR of pH 6.5 (Table 16).

Group 3 (IgA isotype, LPS specific, non cross-reactive) contains nine Mabs which exhibit a range of reactivities with SL/BR. All the antibodies in this group reduce the pseudo-isoelectric point (mean: pH 5.8, sd: 0.35) (Table 16).

Group 4 (non IgA isotype, LPS specific, non cross-reactive) has four Mabs, 3 of the IgM isotype and 1 IgG1. The IgG1 antibody (7/8/64) elevated the pseudo-isoelectric point of treated SL/BR increasing it to pH 7.3 compared with pH 6.9 for controls. Although the IgM antibodies lowered the pseudo-isoelectric point of treated SL/BR no significant difference was seen between the individual monoclonal antibodies (mean: 6.5, sd: 0.32) (Table 16).

3.7.7. Correlation of bacterial surface hydrophobicity of monoclonal antibody (Mab) treated Salmonella typhimurium SL3261 BRD026 (SL/BR) and the inhibition of adherence to Madin-Darby canine kidney (MDCK) cell-line.

The results of correlating the level of inhibition of adherence by the monoclonal antibodies of group 1 to 4

with their effects on the hydrophobicity of treated SL/BR are shown in Fig 24.

As can be seen from the graph the various isotypes appear to cluster in 3 distinct groups. When a best-fit straight line was applied to the data a correlation coefficient of 0.74 was produced but on further analysis it was found that by removing the IgG1 antibody data point the correlation coefficient was reduced to 0.12. Correlating the IgA and IgM antibodies as discrete groups also reveal poor correlation coefficients (0.03 and 0.15, respectively).

3.7.8. Correlation of bacterial net surface charge of monoclonal antibody (Mab) treated Salmonella typhimurium SL3261 BRD026 (SL/BR) and the inhibition of adherence to Madin-Darby canine kidney (MDCK) cell-line.

The results of correlating the level of inhibition of adherence by the monoclonal antibodies of group 1 to 4 with their effects on the negative surface charge of treated SL/BR as assessed by pseudo-isoelectric point are shown in Fig 25.

Curve fitting to produce the best straight line for all the data gives a good correlation coefficient of 0.78. In contrast to the correlation of hydrophobicity with inhibition of adherence the correlation coefficients for IgA and non-IgA groups are also very good (0.72 and 0.93, respectively). There was also a less distinct clustering of isotypes than with the hydrophobicity correlation data.

Figure 24. Correlation of the hydrophobic effect, as measured by hydrophobic interaction chromatography, of monoclonal antibody treatment on S. typhimurium SL3261 BRD026 (SL/BR) with inhibition of adherence to Madin-Darby canine kidney cell (MDCK) monolayers - Groups 1 to 4.

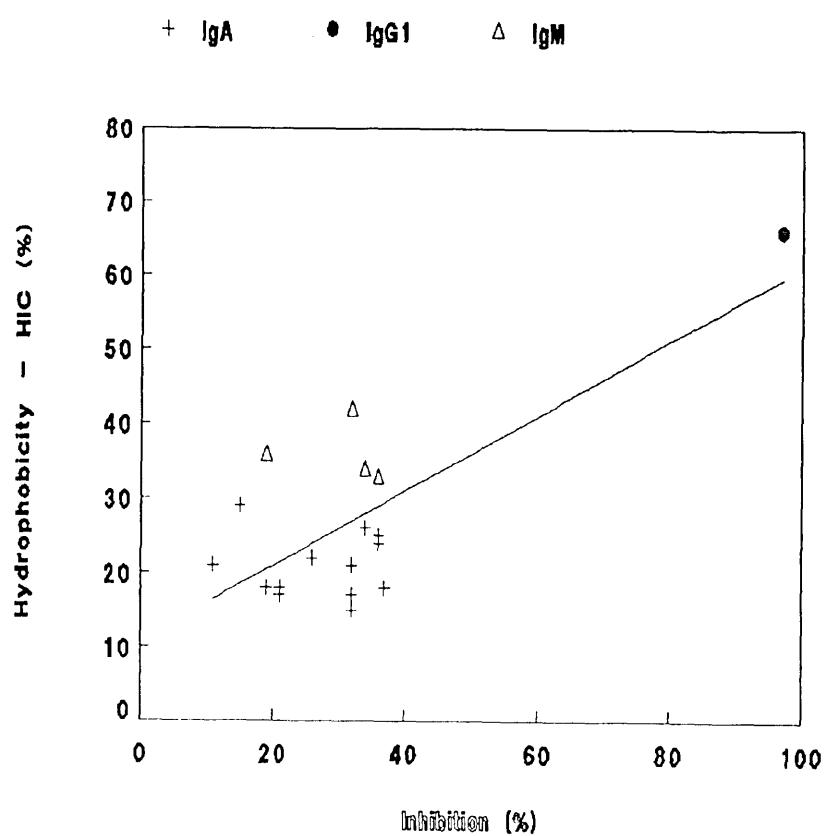
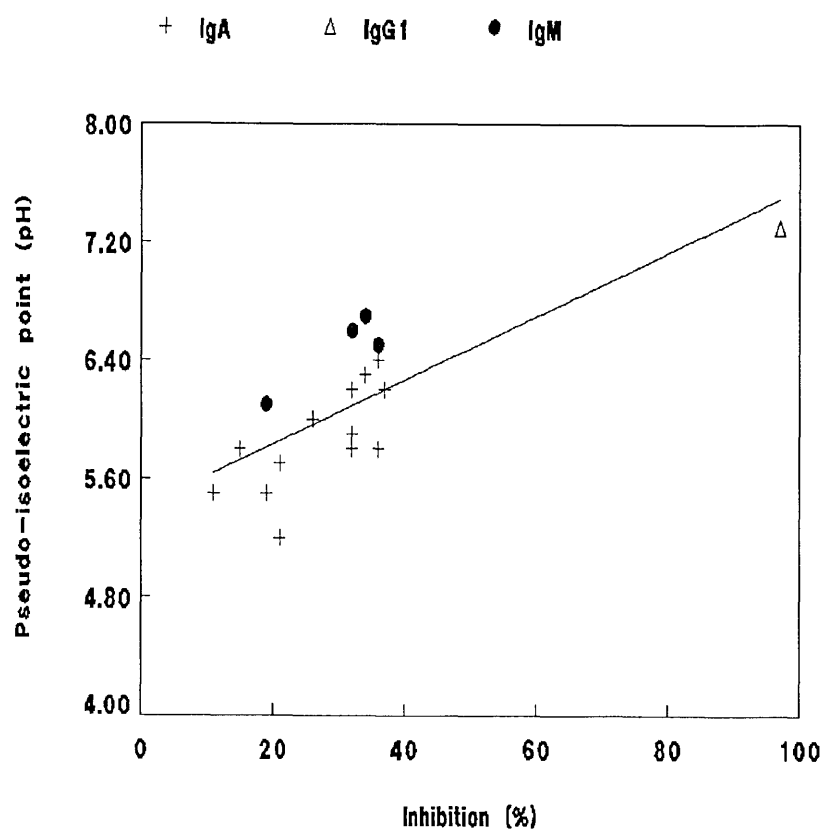


Figure 25. Correlation of the surface charge effect, expressed as pseudo-isoelectric point, of monoclonal antibody treatment on S. typhimurium SL3261 BRD026 (SL/BR) with inhibition of adherence to Madin-Darby canine kidney cell (MDCK) monolayers - Groups 1 to 4.



3.8 ASSESSMENT OF PARAMETERS IN CHEMILUMINESCENCE ASSAY FOR THE COMPARISON OF THE OPSONIC PROPERTIES OF IGA MONOCLONAL ANTIBODIES WITH OTHER ISOTYPES.

Mouse monoclonal antibodies of isotype IgG1, IgM and IgA specific for the capsular polysaccharide of Neisseria meningitidis (type Y) were kindly donated by Dr. R Lifely, Wellcome Biotech.

These Mabs were used to compare the opsonic capabilities of the differing isotypes in a luminol-dependant chemiluminescence (CL) assay (Easmon et al., 1980).

3.8.1. Comparison of opsonic capabilities of IgA, IgM and IgG1 monoclonal antibodies in a chemiluminescence assay.

Opsonization of N. meningitidis (type Y) organisms using human peripheral polymorphonuclear leucocytes (PMNL) was measured for each Mab. A time point for measuring maximum chemiluminescence was to be chosen from these experiments which would be representative for all isotypes.

The time courses of maximum chemiluminescence recorded for each of the isotypes were however different in character, with a graduation in maximum chemiluminescence after a short period greatest for IgG1 then IgM and the least for IgA (See Fig 26).

All isotypes gave an initial rise in chemiluminescence after the first few minutes. IgG1 continued to climb steeply reaching a peak greater than IgM or IgA. It then declined rapidly with almost no chemiluminescence after 10 minutes. IgM gave a slower rise to an intermediate peak less than IgG1 but greater than IgA. This slower rise was followed by a slower decline in chemiluminescence as well. IgA rose to a peak CL much lower than IgG1 or IgM but sustained this level of chemiluminescence with only a slow decline over a very long time period.

Figure 26. Typical chemiluminescence profiles for different isotype of monoclonal antibodies specific for Neisseria meningitidis (type y) capsular polysaccharide: IgG1, IgA and IgM.

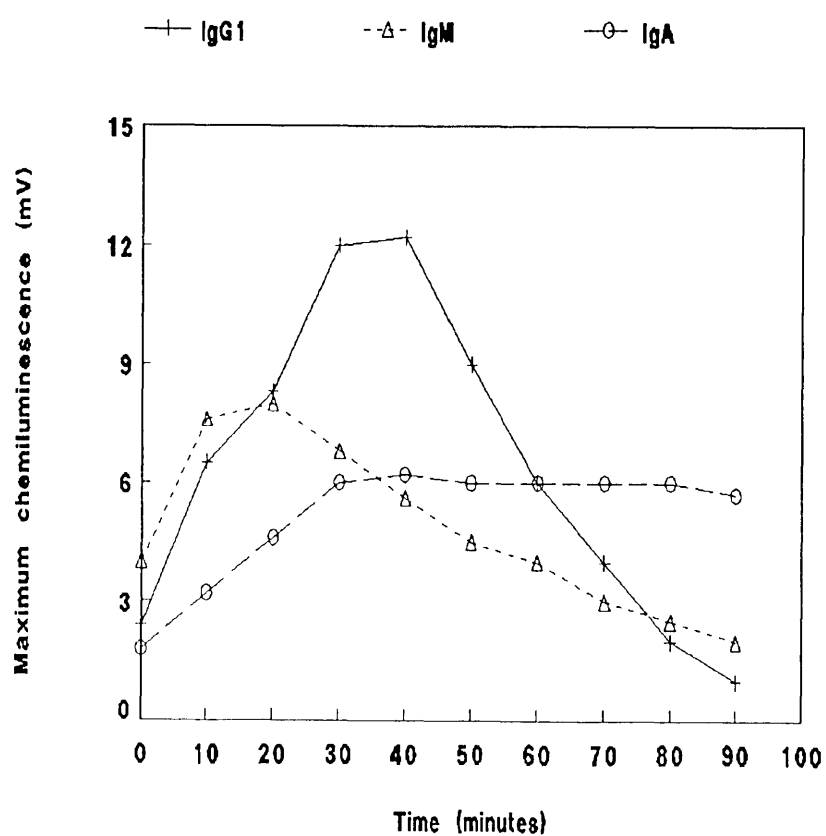
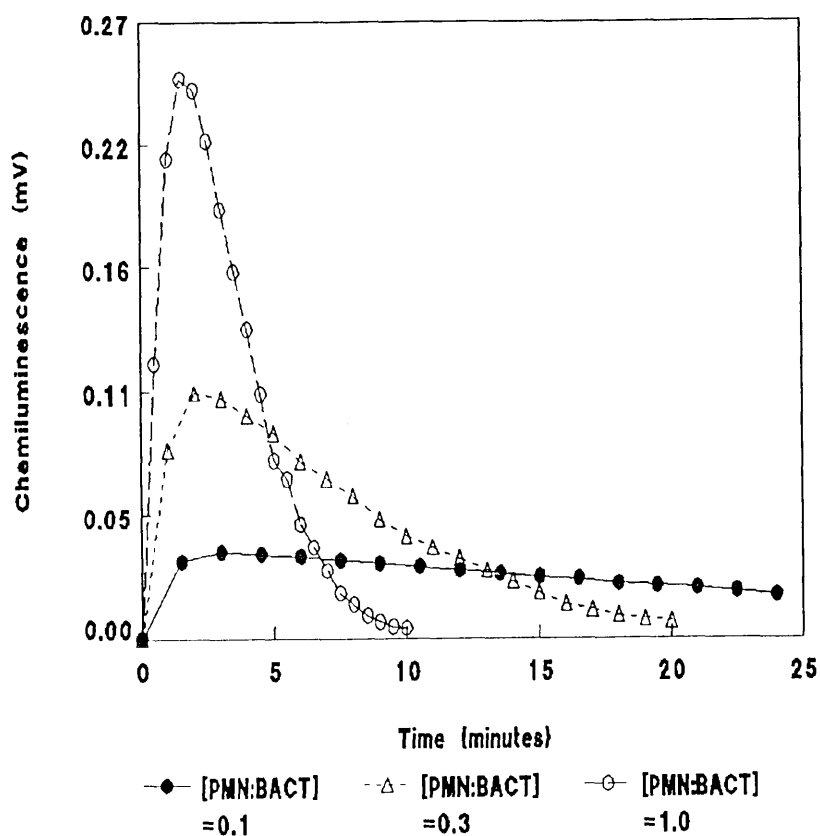


Figure 27. Comparison of chemiluminescent responses within a computer model with varying relative concentrations of polymorphonuclear leucocytes compared with opsonized bacteria.



3.8.2. Computer model of chemiluminescence production by opsonized bacteria.

In order to better understand the cause of this variation in the chemiluminescence profiles of the various isotypes a computer model was developed with which to simulate these profiles. It allowed an assessment of the relative contribution of each of the components of the assay in a simplistic manner so that predictions could be made, and further experiments suggested.

From the model results it could be shown that by increasing or decreasing the number of polymorphonuclear leucocytes (PMN) within the system chemiluminescence profiles similar to those produced by each of the isotypes could be simulated (Fig 27). The model predicted that the chemiluminescence profile for IgG1 varied from that of IgA in that the level of available PMN capable of chemiluminescence was higher for IgG1 than for IgA. This could relate to previous work carried out by Fanger et al. (1980) who reported that more human peripheral blood PMN possess Fc-receptors for IgG than for IgA, 80% carrying IgG receptors and 30% carrying IgA receptors.

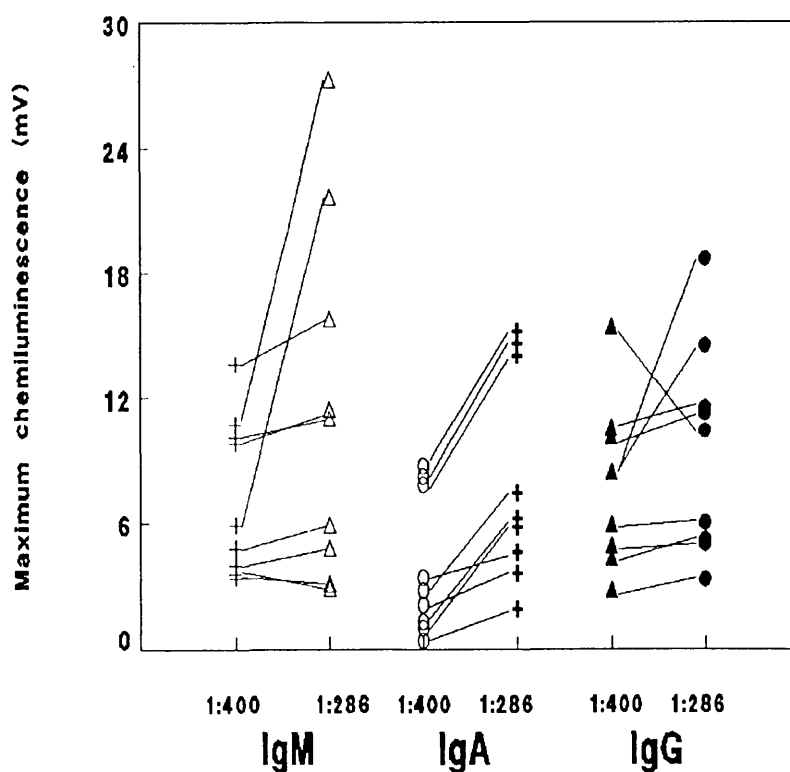
3.8.3. Comparison of the effect of the ratio of Polymorphonuclear leucocytes to bacteria on chemiluminescence production using IgG1, IgA and IgM as opsonins.

A further series of experiments were carried out in which the concentrations of PMN within the assay system was altered to give two different ratios of PMN:opsonized bacteria. The computer model predicted that the rise in the ratio of PMN:opsonized bacteria would result in a rise in the peak value of chemiluminescence for IgA opsonized bacteria between the two systems. A less significant rise would be seen with the IgG1 opsonized bacteria.

The ratios were 1:400 (as in the original experiments) and 1:286, PMN:opsonized bacteria respectively.

The experiment used blood from 8 donors with one donor volunteering twice. The results are shown in Fig 28 and show that although there was a small relative increase for IgG1 it was not significant. IgM showed a greater increase than with IgG1 but this was not significant. IgA showed an increase for each donor and overall gave a significant

Figure 28. Comparison of maximum chemiluminescent responses of human polymorphonuclear leucocytes to Neisseria meningitidis opsonized by IgG1, IgA and IgM monoclonal antibodies directed against capsular polysaccharide with differing ratios of polymorphonuclear leucocytes to opsonized bacteria.



increase ($p < 0.01$) when using a paired t-test to analyze the data.

4 . DISCUSSION .

4.1 PRODUCTION OF IGA MONOCLONAL ANTIBODIES.

The use of monoclonal antibodies has greatly facilitated the investigation of humoral systemic immunity, but conventional techniques have been unable to produce IgA secreting hybridomas in any great numbers (Dean et al., 1986). Studies of the biological role of IgA antibodies in humoral mucosal immunity have been hampered by the lack of a defined population of these molecules with the capacity to bind a specific antigen. Most hybridomas derived from spleen cells by the standard fusion protocols of Kohler and Milstein (1975; 1976) secrete antibodies of either the IgM or IgG classes.

Recent developments have meant that there are now techniques available for the production of primarily IgA secreting hybridomas with lymphocytes isolated from mucosal tissue following mucosal immunization. Workers have shown that after allogeneic cells (Hall et al., 1979) or syngeneic sarcoma cells (Gyure et al, 1980) are injected into the Peyer's patches of rats, specific antibodies of the IgA class are formed in the mesenteric nodes and appear in the bile. By taking cells from the mesenteric lymph nodes of rats immunized in this way and fusing them with cells of the Y3.Ag. 1.2.3 rat myeloma (Galfre et al. 1979) they obtained and cloned rat

hybridomas which secreted antibodies of the IgA class (Dean et al., 1984a).

These techniques were used to prepare 32 hybridomas secreting monoclonal antibodies directed against S. typhimurium, an important enteric pathogen. Of these hybridomas, 20 secreted IgA, 11 secreted IgM and 1 secreted IgG1. These monoclonal antibodies were assessed for their ability to prevent initial infection using an in vitro adherence model with Madin Darby canine kidney (MDCK) epithelial cell monolayers.

The 32 hybridomas were isolated following 4 fusions, 2 of which used primed lymphocytes from mesenteric lymph nodes (MLN) and 2 of which used primed lymphocytes from efferent mesenteric lymph (EML). No significant difference was seen in the proportion of IgA secreting hybridomas isolated when using mesenteric lymph nodes and efferent mesenteric lymph as a source of primed lymphocytes for fusion. EML produced 26 selected hybridomas of which 65% were IgA secreting. MLN produced only 6 selected hybridomas of which 67% were IgA secreting.

These results are at odds with the finding of Dean et al. (1984) and Styles et al. (1984) who reported that EML was superior to MLN in the production of IgA secreting hybridomas. Dean et al. (1984) found that the mesenteric nodes of rats stimulated by injecting antigen into the Peyer's patches, were an excellent source of antibody producing cells suitable for use in hybridoma production. Hybridomas secreting antibodies of most

isotypes could be obtained from this source and would be of value where studies of immunoglobulin class function were being undertaken. However, although IgA secreting hybridomas were obtained with this protocol their frequency of production was substantially lower than that of other isotypes (Dean et al., 1984).

It is clear that mesenteric nodes contain IgA producing cells (Andrew & Hall, 1982) but Dean and his colleagues speculated that the majority were too far along the differentiation pathway to plasma cells to be able to undergo successful fusion with the Y3 myeloma. Certainly, the results were consistent with the findings of others (Kawanishi et al., 1983a, 1983b) that following the switch from sIgM to sIgA production in the Peyer's patches the IgA committed B cells migrate to the mesenteric nodes where they differentiate further. Indeed, specific IgA plaque forming cells can be found in the mesenteric nodes following injection of antigen into Peyer's patches (Andrew & Hall, 1982).

Their results supported the view that the mesenteric nodes were not strictly part of the GALT but like other regional lymph nodes were largely concerned with the generation of cells that form IgM or IgG and the small number of IgA producing cells were a consequence of immigration from the wall of the gut.

Styles et al. (1984) used the efferent mesenteric lymph, collected by canulation of the cisterna chyli following previous removal of the mesenteric lymph node as a method whereby the intestinal peripheral lymph, that

would normally drain into the mesenteric lymph nodes, could be used as a source of primed lymphocytes for fusion (Styles et al., 1984). This was found to be a better source of IgA producing cells suitable for fusion (Styles et al., 1984). Most of the plasma cells in the lamina propria of the rat intestine make IgA (Husband, Monie & Gowans, 1977) so it is not unexpected to find that large numbers of cells suitable for the preparation of hybridomas secreting IgA are present in peripheral intestinal lymph. EML was also used in this project for fusion and provided a large number of hybridomas (26) with a majority of IgA secreters (67%) consistent with the results of Styles et al. (1984)

The difference in the findings of Dean et al. (1984) and this project may be related to the cells used for immunization. They used a syngeneic tumour cell line whose immunodominant antigen would be only poorly immunogenic and novel being previously unseen by the lymphocyte pool of the Peyer's patches (North et al., 1982). The immunodominant antigen of S. typhimurium SL/BR was the O-antigen of lipopolysaccharide (LPS) a naturally occurring antigen in the gastrointestinal tract (Lindberg, 1980). Naturally occurring enteric antigens have been shown to induce an IgA-class restricted response, whilst virgin novel antigens produce equal numbers of IgG, IgM and IgA secreting B-cell clones (Gearhart and Cebra, 1979; Cebra et al., 1976, 1983). This may account for the increased IgA response seen with

MLN compared with the results of Dean et al. (1984) and is further discussed in the next section.

The number of monoclonal antibodies of IgA isotype specific for LPS was approximately 2.5 fold greater than those of non-IgA isotype. In comparison, equal numbers of monoclonal antibodies of both IgA and non-IgA isotype were directed against other bacterial antigens. It would appear from this that in the selected monoclonal antibodies there was a bias in the isotype expression of anti-LPS antibodies. The role of LPS in the stimulation of mucosal immune responses has been investigated by a number of workers. LPS can stimulate the non-specific activation and proliferation of immature B-cells (McGhee et al., 1984).

In the enteric environment where the levels of LPS are routinely high there may be a predisposition for B-cells committed to IgA secretion to be induced by LPS, perhaps as a means of producing local tolerance. PP contain higher frequencies of B cells committed to differentiation into IgA plasma cells than any other lymphoid tissue (Craig and Cebra, 1971, 1975). Experiments using clonal analysis of PP B cells have shown that 40%-60% of clones with specificity for naturally occurring enteric haptens such as phosphocholine, inulin and beta-galactose exclusively express IgA, while B cells of virgin antigen specificities such as anti-dinitrophenyl, yielded equal mixtures of IgM, IgG and IgA. These latter clones were similar to those obtained from the spleens of the same

animals (Gearhart and Cebra, 1979; Cebra et al. 1983).

This analysis strongly suggests that a selective priming of PP B cells occurs by naturally encountered antigens and results in subsequent restriction of the isotype potential of B cells to IgA expression (Cebra et al., 1984). The results of this project would support the idea that there is a class restriction in the response to LPS, a naturally occurring enteric antigen.

Nine of the 13 selected IgA monoclonal antibodies specific for lipopolysaccharide appeared to produce a variety of molecular weight forms as assessed by non-reducing SDS-PAGE. These IgA Mabs produced three forms of molecular weight 360 kD, 320 kD and 160 kD. The other 4 only produced one molecular form with a molecular weight of 360 kD.

Two IgA Mabs (PIP18 and PIP2) which produced multiple molecular forms underwent a process of separation to produce preparations of all three of the molecular forms. Each Mab produced 3 preparations which corresponded to the 360 kD, 320 kD and 160 kD molecular forms as described above. The molecular weight and purity of each molecular form was confirmed by non-reduced SDS-PAGE.

The 160 kD form would appear to be a monomeric form of IgA whereas the 360 kD and 320 kD forms are both close to the molecular weight of the dimeric form of IgA. The presence of a dimer at the approximate dimeric molecular weight of IgA could be related to the presence or absence

of J-chain, a protein of approximate molecular weight 20-30 kD which is normally found associated with dimeric IgA. The 360 kD form may represent the dimeric IgA with J-chain, whilst the 320 kD form could be the result of the loss of J-chain from the 360 kD form either by proteolytic digestion or possibly through some error in the processing of the dimeric IgA by the hybridomas involved.

Of 18 monoclonal antibodies specific for Salmonella typhimurium lipopolysaccharide, 5 were found to be cross-reactive with lipopolysaccharide from Salmonella minnesota. The cross-reactivity as assessed by RIA was far less than the initial reactivity for S. typhimurium LPS.

Cross reactivity due to LPS core antigens was ruled out as none of the monoclonal antibodies were reactive with LPS preparations from mutant strains of S. minnesota which produce rough LPS devoid of the O antigen side chains. The actual length of the core structure in these strains differed with the preparation. Three chemotype rough LPS preparations were used. The Ra-chemotype containing the complete region. The Rc chemotype which is intermediate in length, containing approximately half the core region and that with the shortest core region, termed the Re-chemotype. The cause of the cross-reactivity is unclear as the two strains appear to possess different O-antigens.

As there are no similarities in the primary sequence of the saccharide units of the two strains (Hellerqvist

et al., 1969; Luderitz et al., 1966), one possible explanation for the cross-reactivity may be that there are antigenic similarities between the O-antigens of the two strains which are conformational in character. The chemical structure of the immunological determinants of S. typhimurium LPS have been extensively characterized. The structures responsible for the O-antigenic determinant of S. minnesota are still unclear, as quantitative immunochemical studies have not been done (Lindberg and Le Minor, 1984), so preventing comparison with S. typhimurium LPS.

4.2 ASSOCIATION OF SALMONELLA TYPHIMURIUM SL3261 BRD026 (SL/BR) WITH MADIN DARBY CANINE KIDNEY (MDCK) CELL MONOLAYERS.

The underlying mechanisms used by Salmonella to attach to and penetrate host epithelia are not well understood (Moulder, 1985). Part of the difficulty has been the lack of suitable in vitro methods to study epithelial cell invasion. Most current knowledge has accumulated from animal studies.

In vivo, the first intestinal epithelial surface encountered by ingested Salmonella is the apical surface. Only later in infections are Salmonella observed near the basolateral surface of epithelial cells, often in the underlying lamina propria (Takeuchi, 1967). Perhaps Salmonella interact predominantly with apical surfaces or

a receptor located primarily on the apical surface, producing a directionality during infection in vivo.

Takeuchi (1967) produced a comprehensive descriptive study of Salmonella tissue penetration using transmission electron microscopy to examine the intestinal epithelium of guinea pigs orally infected with Salmonella typhimurium. Once the bacteria came into close proximity to the brush border, the epithelial microvilli began to degenerate. Most of the bacteria entered directly into the epithelial cells and resided within membrane bound cavities. A few bacteria appeared to enter at intracellular junctions and remained surrounded by a vacuole composed of neighbouring epithelial cells. Neither bacterial exit from the epithelial cells nor entry into the underlying lamina propria was observed.

In vitro tissue culture models have also been used to examine initial interactions and internalization of S. typhimurium and S. typhi into HeLa and HEp-2 cells (Ciannella et al, 1978; Kihlstrom and Nilsson, 1977; Yabuuchi et al., 1986; Yokoyama et al., 1987). Internalization of salmonellae into these cells appeared to proceed by a similar mechanism to that observed with the guinea pig model.

Finlay and Falkow (1988) investigated the events that lead to salmonella invasion by examining the interaction of Salmonella choleraesuis, a common swine pathogen which is also a highly invasive and serious human pathogen (Rubin and Weinstein, 1977), with Madin-Darby canine kidney (MDCK) cells. This is a polarized

epithelial cell line isolated from the kidney of a cocker spaniel (ATCC, 1981).

These cells form a polarized epithelial monolayer when grown on a permeable support which is impermeable to ions, has a measurable transepithelial electrical resistance and has several defined apical and basolateral surface markers (reviewed by Simons and Fuller, 1988). Physiologists have long used this system for studying epithelial transport.

This in vitro system was similar in many respects to animal infection models.

Loss of intestinal epithelial microvilli after salmonella infection occurs in mice ileal loops (S. typhi; Kohbata et al., 1986) and in the guinea pig ileum (S. typhimurium; Takeuchi, 1967). Takeuchi reported uniform penetration of the guinea pig intestinal epithelium by S. typhimurium. These bacteria were internalized within membrane bound inclusions and remained within these vacuoles. After 24 hours bacteria were observed within phagocytes in the underlying lamina propria indicating epithelial penetration.

Apical S. choleraesuis infection of polarized MDCK monolayers demonstrated all of these qualities, making MDCK cells a very attractive in vitro model to study the precise mechanisms of salmonella invasion of epithelial cells.

Finlay and colleagues, also found that 10-fold more S. choleraesuis associated with the apical surface than the basolateral surface after 4 hours of infection and

that this increased to 23-fold after 6 hours. Bacterial penetration was also 100-fold greater using apically infected cells. It appeared that S. choleraesuis interacts predominantly with the apical surface of the MDCK cell, another feature common in natural epithelia.

Further, the association between S. choleraesuis and MDCK monolayers was not a simple "receptor-ligand" association but was an active process. Use of inhibitors of bacterial protein synthesis even when the drug was only bacteriostatic led to prevention of invasion by S. choleraesuis.

During the adherence assay, viable S. typhimurium SL/BR were capable of replication. The level of S. typhimurium SL/BR replication within EMEM was such that with an initial inoculum of 50 colony forming units of S. typhimurium SL/BR in exponential growth there was an initial lag phase for the first 3 hours. After 5 hours the culture was once again in exponential growth, which continued until the cultures were terminated after 7 hours.

These results demonstrated that the increase in bacteria within the adherence assay system over the first 3 hours would be negligible. During this time these bacteria would be readjusting to the static, microaerophilic conditions of the assay system which would more closely resemble the conditions in vivo.

Varying the initial inoculum size of S. typhimurium SL/BR had little or no effect on the time course of adherence to the MDCK cell monolayers. All inocula

followed the same time course for adherence in a dose-dependant fashion with a lag phase between 0 and 3 hours after which the rate of adherence became exponential. The initial lag phase could not be overcome by using progressively higher inoculum sizes implying that the levels of S. typhimurium SL/BR bound to the MDCK monolayer was only dependant on the period of incubation.

The ratio of bound to unbound bacteria was relatively constant for all inoculum sizes suggesting that adherence was dependant on the bacteria rather than on the presence of a number of discrete sites for adherence on the MDCK monolayer. If there were only a discrete number of sites on the monolayer to which S. typhimurium SL/BR could adhere, then use of progressively higher inocula would lead to a saturation effect such that once all the sites were occupied any increase in the inoculum size would result in a reduction in the ratio of bound to unbound bacteria. This was not seen.

It would appear from this that specific receptor-adhesin interactions were not taking place and that the attachment of S. typhimurium SL/BR bacteria to MDCK cell monolayers was more than a simple "receptor-ligand" association.

Lipopolysaccharide (LPS) is exposed on the surface of S. typhimurium bacteria and is therefore a possible candidate for involvement in bacterial adherence to animal cells (Lindberg, 1980). LPS extracted from gram-negative bacteria has by itself an affinity for animal cells (Kabir et al., 1978).

Extracted LPS exists as a bilayer with the hydrophobic lipid moieties buried in the centre and the hydrophilic polysaccharides exposed on the surface (Shands, 1971). LPS associates with animal cell membranes through hydrophobic and charge interactions (Benedetto et al., 1973) rather than through a specific receptor.

In these experiments an S. typhimurium (WT) LPS preparation was found to have no effect as an inhibitor of the adherence of S. typhimurium SL/BR to MDCK cell monolayers.

The fact that the LPS preparation could not antagonize bacterial adherence indicates that there are no discrete bacterial adherence-areas with affinity for LPS on the MDCK cell surface.

Interestingly, there appeared to be an increase in the adherence of S. typhimurium SL/BR with increasing concentrations of LPS as compared with controls to which only PBS was added. The increase was not significant but the results show a clear trend. The association of LPS with animal cells may mean that LPS is binding to the MDCK cell surface resulting in changes in its physico-chemical characteristics or perhaps the induction of changes in the MDCK cell metabolism which result in bacterial attachment and penetration.

Since LPS is released from viable and disintegrating salmonella and has affinity for animal cell surfaces it is possible that LPS may promote bacterial adherence at mucosal surfaces.

4.3 INHIBITION OF ADHERENCE OF SALMONELLA TYPHIMURIUM SL3261 BRD026 (SL/BR) TO MADIN DARBY CANINE KIDNEY (MDCK) CELL MONOLAYERS BY TREATMENT WITH MONOCLONAL ANTIBODIES.

All the monoclonal antibodies specific for LPS were capable of inhibiting the adherence of S. typhimurium SL/BR to MDCK cell monolayers except for the single IgG1 antibody.

Pre-incubation of monoclonal antibody and bacteria for up to 60 minutes had no effect on the adherence of antibody treated bacteria as measured after 3 hours. All monoclonal antibodies, including the IgG1 antibody, showed a slight increase in initial inhibition of adherence at time 0 hours when pre-incubated for 60 minutes. This increase was not significant. Monoclonal antibody binding to S. typhimurium SL/BR in the liquid phase of the adherence assay would appear to be rapid and effective.

Post-addition of the monoclonal antibodies after the start of the assay was found to be ineffective after 2 hours giving only slight reductions in adherence compared with controls. Addition of the monoclonal antibody was necessary between 0 and 1 hours to produce any significant inhibition with the highest levels attained with addition at the start of the assay (0 hours), as in the initial assessment experiments. This lack of effect as time progresses would argue that S. typhimurium SL/BR may bind reversibly at first, so being susceptible to inhibition by monoclonal antibodies at the start of the

assay. However as the results demonstrate, the binding quickly becomes irreversible after 2-3 hours.

Further experiments showed that the monoclonal antibodies had no effect on bacteria already adherent to the MDCK monolayer. Addition of monoclonal antibodies to monolayers previously incubated with S. typhimurium SL/BR for 3 hours produced no immediate effect. Subsequently the levels of adherence increased over a further 3 hours in parallel with controls. After the 6 hours no reduction in the levels of the initial bound bacteria had been seen but there was a relative reduction in adherence compared with controls.

These results all combine to demonstrate that S. typhimurium SL/BR appears to undergo a progressive change from a reversible to an irreversible binding to MDCK cell monolayers over the period of 3 hours. The success of the IgA and IgM monoclonal antibodies in preventing adherence would appear to occur at the early reversible binding stage with little effect being seen once the bacteria were adherent on the MDCK cell monolayer.

The IgG1 antibody (7/8/64) was the only antibody which did not inhibit adherence of S. typhimurium SL/BR to MDCK cell monolayers. In order to investigate whether it would actively compete with the other isotypes to reduce their inhibitory effect two IgA antibodies from group 1 and 3, and two IgM antibodies from group 2 and 4 were titrated individually in competition with 7/8/64.

The level of IgG1 antibody was held at the ABT^{50%} concentration, the dilution at which the antibody gave

50% binding in solid phase radioimmunoassay. The other antibody was titrated from 4 $TI^{50\%}$ to 0.125 $TI^{50\%}$, where $TI^{50\%}$ is equal to the dilution at which the antibody produced 50% inhibition of adherence of S. typhimurium SL/BR to MDCK monolayers. The arrangements were then reversed and the inhibitory antibody was added at $TI^{50\%}$ concentration with the IgG1 antibody titrated from 4 $ABT^{50\%}$ to 0.125 $ABT^{50\%}$.

Titration of the IgG1 antibody against both IgA and IgM antibodies produced similar results with a reduction in the inhibitory effect of the IgA and IgM antibodies when titrated with IgG1 at a fixed concentration. Conversely when titrating IgG1 with the IgA and IgM antibodies there was an increase in the inhibition of adherence as the IgG1 concentration declined.

It appears that the IgG1 antibody had a competitive effect on the inhibition of adherence to MDCK cell monolayers seen with IgA and IgM treated S. typhimurium SL/BR. At high relative concentrations the IgG1 monoclonal antibody will reduce the inhibitory effect seen with the IgA and IgM monoclonal antibodies, though the level of this reduction appears to differ for IgA and IgM. The point at which the adherence was the same for both titrations (described as the "equivalence point") was different for IgA and IgM.

For the IgA monoclonal antibodies the equivalence point of the titration curves occurred at approximately 0.5 $TI^{50\%}$, whilst the IgM monoclonal antibodies displayed a cross-over point of approximately 1.0 $TI^{50\%}$.

It is assumed that these "equivalence points" represent a situation at which there are equal quantities of IgG1 and either IgA or IgM bound to the available S. typhimurium SL/BR.

With the IgA monoclonal antibodies the equivalence point corresponded to that point at which there were equal quantities of both IgA and IgG1. But with the IgM monoclonal antibodies the equivalence point corresponds to the point at which there is approximately a 2-fold greater concentration of IgM compared with IgG1. If this is so then a lower concentration of IgA is required to produce equal binding with IgG1 than with IgM.

In order to reconcile this difference a model may be proposed in which both IgG1 and IgA have the same binding ability in terms of avidity and binding constants. The $TI^{50\%}$ for IgG1 was based on its $ABT^{50\%}$ as it did not inhibit adherence. It is possible that in liquid phase, the $ABT^{50\%}$ for IgG1 may be lower and a comparable $TI^{50\%}$ can be assumed to be that dilution at which the equivalence point occurs with the IgA monoclonal antibodies.

If these assumptions are made then the higher equivalence point for the IgM monoclonal antibodies could be explained by proposing a lower binding ability when compared with IgG1. In this situation an equivalence point would only be reached where IgM was at a greater concentration than IgG1.

A similar situation was reported by Griffiss and Goroff (1983) with IgA blockade of IgM-initiated immune

lysis of Neisseria meningitidis. Whereas IgA blockade of IgG-initiated immune lysis was a competitive function of the ratio of IgA to IgG, the blocking of IgM-initiated immune lysis was a non-competitive function of the ratio of IgA to bacterial cells, independent of the concentration of IgM.

In the presence of sufficient IgA to saturate available binding sites IgM was an impotent bystander unable to compete for sites and initiate lysis. This non-competitive exclusion of IgM from the surface of bacterial cells may occur with S. typhimurium SL/BR and so result in the difference seen in equivalence points described above.

Amiloride has been shown to be a potent inhibitor of sodium transport by intact epithelia in vitro (Bentley, 1968). Bentley first described the effect of amiloride on toad bladder epithelia. Cereijido et al. (1978) demonstrated that, when used at concentrations capable of inhibiting sodium transport in natural epithelia, amiloride could reduce the electrical potential produced by intact MDCK cell monolayers without affecting cell viability.

MDCK cell monolayers possess a high electrical potential based on selective sodium transport which results in the apical surface carrying a large negative surface charge when compared with the basolateral surface. Although the effect of addition to natural epithelia was temporary and reversible, once treated MDCK cell monolayers underwent a constant reduction of their

electrical potential which was unaffected by time or subsequent washing.

The experiments performed here used concentrations of amiloride ranging from that which had been shown to have no effect on the electrical potential of the MDCK monolayers (10^{-8} M) to that which had been shown to destroy the electrical potential entirely (10^{-5} M) (Cereijido et al., 1978).

The adherence of S. typhimurium (SL/BR) to MDCK cell monolayers was not significantly inhibited by increasing concentrations of amiloride although there was a trend for a small reduction in adherence with increasing amiloride concentration. This reduction was not significant. In contrast increasing amiloride concentrations were seen to have an effect on the adherence of the majority of monoclonal antibody treated S. typhimurium SL/BR.

Increasing amiloride concentrations resulted in a reduction of the inhibitory effect of the IgA and IgM antibodies of Groups 1 to 4 in a dose dependant fashion. S. typhimurium SL/BR treated with the IgG1 antibody (7/8/64), which had no inhibitory effect, acted in the same fashion as the untreated bacteria giving a slight reduction in adherence with increasing Amiloride concentration.

The inhibition of adherence correlated with the increase in amiloride concentration. The increasing amiloride concentration would cause a reduction of the electrical potential of the MDCK cell monolayer and so

reduce the negative charge on the apical surface of the monolayers. If the inhibition of adherence of S. typhimurium (SL/BR) treated with IgA or IgM monoclonal antibodies were caused by increased charge repulsion, it is possible that by reducing the negative charge on the apical surface of MDCK cell monolayers the inhibitory effects of the monoclonal antibodies would be decreased.

These results suggested that this might be the case and later experiments confirmed this idea.

4.4 PHYSICOCHEMICAL PROPERTIES AS RELATED TO BACTERIAL INTERACTION WITH ANIMAL CELLS.

The properties of a bacterial cell surface which determine the tendency to interact with an animal cell surface are only poorly understood. However, charge and hydrophobicity of the bacterial cell surface has been shown to have a profound effect on their ability to adhere to animal cells (Albertsson, 1987).

Animal cells are by nature hydrophobic and carry a negative surface charge. In addition MDCK cell monolayers, in common with other natural epithelia (eg gastrointestinal tract) are capable of producing an electrical potential with an increased negative charge on the apical surface when compared with the basolateral surface.

Hydrophobic interaction, or the hydrophobic effect, is a chemical reaction between two or more substances or particles in aqueous phase with the elimination of the

water associated with each particle. Since the state of separate particles surrounded by water is more energy requiring than the bound state, a gain in free energy results.

The hydrophobicity of the bacterial cell surface has been demonstrated by partition in aqueous polymer biphasic systems (Stendahl et al., 1973; Colleen et al., 1979; Gerson and Akit, 1980; Rosenberg et al., 1981), by hydrophobic interaction chromatography (Smyth et al., 1978; Perers et al., 1977; Kihlstrom and Edebo, 1976; Tylewska et al., 1979 1980) or by adhesion of bacteria to hydrophobic plastic surfaces (Gerson and Scheer, 1980).

In general, hydrophobic surface properties of bacteria seem to promote their association with different animal cells. As bacteria approach host cell surfaces, hydrophobic molecules present on the two cell surfaces can overcome any increasing repulsive forces created by their individual negative charges.

The hydrophobicity of S. typhimurium SL/BR treated with monoclonal antibodies was assessed by partition between an aqueous phase and an octan-1-ol phase to analyze any difference in the surface properties. This method was subject to large variation between assays and so another method was employed using hydrophobic interaction chromatography (HIC).

HIC confirmed the results obtained with octan-1-ol:aqueous partitioning and gave less variation between assays allowing for greater reproducibility. Both methods found S. typhimurium SL/BR itself to be relatively

hydrophilic with a hydrophobicity percentage of 36% (sd: 3.4%) with octan-1-ol partitioning and a hydrophobicity index of 35% (sd: 2.1%) using HIC.

The effect on the hydrophobicity of S. typhimurium SL/BR when treated with monoclonal antibodies was very much isotype dependant. Treatment with IgA monoclonal antibodies gave a universal decrease in the hydrophobicity of S. typhimurium SL/BR, whilst IgM had no significant effect. The one IgG1 antibody gave a very large significant increase in the hydrophobicity of S. typhimurium SL/BR.

The difference in hydrophobicity of the monoclonal treated S. typhimurium SL/BR could be a factor in explaining the variation in inhibitory effects of the different isotypes, specifically IgA and IgM as compared with the one IgG1 antibody. Decreases in bacterial surface hydrophobicity have been shown to reduce their ability to adhere to animal cells.

Perers et al. (1977) demonstrated that the more hydrophobic strains of Salmonella typhimurium and E. coli adhered significantly better to mouse intestinal mucosa in vivo than relatively hydrophilic strains of the same organisms.

Smyth et al (1978) also showed that K88-antigen positive strains of E. coli that adhered well to host cells bound strongly to hydrophobic ligands on agarose gels. This contrasted with the weak hydrophobic binding of the isogenic K88-negative mutant, which failed to adhere to host cells.

Miorner et al. (1980) demonstrated that the surface hydrophobicity of several gram-positive bacteria was significantly altered by the adsorption to the organisms of various serum proteins. The adsorption of serum albumin to group A streptococci has been shown to block their attachment to epithelial cells (Beachey et al., 1981).

IgG1 has been shown to possess a hydrophobic character as assessed by contact angle measurements such that when bound to cells or particles it can increase their hydrophobicity and encourage their attachment and engulfment by phagocytic cells in the systemic circulation.

Conversely, IgA has been shown to possess a hydrophilic character as assessed by contact angle measurements and can result in a decrease in the hydrophobicity of those antigens to which it binds. This decrease can result in a reduction in the binding of antigen-antibody complexes to phagocytic cells and has been quoted as a method whereby IgA can inhibit phagocytosis.

IgM has an intermediate hydrophobic character which is very similar to that of the phagocytic cells themselves and so would neither encourage or discourage attachment when bound to antigen. Adherence to phagocytic cells as a prelude to phagocytosis is comparable to the initial attachment of S. typhimurium SL/BR to MDCK cell monolayers.

The change in hydrophobicity caused by the treatment with monoclonal antibodies presents the possibility that this is the most important feature of the effects of the individual isotypes in the adherence assay. Indeed when correlating the inhibitory effect with the change in hydrophobicity the data gave a correlation coefficient of 0.74. But on further analysis it was found that by removing the IgG1 antibody data point the correlation coefficient was reduced to 0.12, and correlating the IgA and IgM antibodies as discrete groups also reveal poor correlation coefficients (0.03 and 0.15, respectively).

Both IgA and IgM monoclonal antibodies produce similar inhibition but IgA decreases hydrophobicity whilst IgM appears to have no significant effect. It would seem that although hydrophobicity may have an effect on the adherence of S. typhimurium SL/BR to MDCK cells there must be some other explanation for the inhibitory effects of both IgA and IgM.

Charges on the surfaces of most bacteria and of the host cells to which they become attached are both, in sum, negative, thereby creating repulsive forces between the cells. These repulsive forces may be overcome by both long-range and short-range attractive forces (Curtis 1973).

Physical studies of negatively charged colloidal particles have indicated that the energy of interaction between two negatively charged particles is the sum of the electrostatic energy of attraction and the energy of repulsion through London-van der Waals forces.

According to this concept, called the DLVO theory (Watt and Ward, 1980), two surfaces, although negatively charged, may attract each other through London forces created by fluctuating dipoles of similar frequency caused by atomic and molecular vibrations on each surface. Simply stated, the repulsive forces decrease more rapidly than do the attractive forces as the distance between the charged surfaces increases; therefore, the long-range forces add up to weak attraction (Curtis, 1973).

The pseudo-isoelectric point was used as a measure of the surface charge of the Mab treated bacteria in order to determine if charge variation would account for the inhibitory effects of the IgA and IgM Monoclonal antibodies.

All of the monoclonal antibodies analyzed increased the net negative surface charge of S. typhimurium SL/BR except for the IgG1 antibody which was the only one which did not inhibit S. typhimurium SL/BR adherence to the MDCK cell monolayers.

There is evidence that changes in the surface charge characteristics of bacteria can either enhance or reduce their ability to adhere to animal cells.

Heckels et al. (1976) demonstrated that a modification of the surface charge of bacteria could change their adherence capacity; blocking of the negatively charged free carboxyl groups on the surfaces of nonfimbriate gonococci increased their adherence ability to the level of fimbriate organisms. On the other

hand, when the negative charges were removed from the target erythrocytes, fimbriate gonococci enjoyed no advantage over nonfimbriated organisms in causing heamagglutination (Watt and Ward, 1980). One of the functions of the fimbriae appeared to be to bridge the electrostatic repulsive barrier between the bacteria and host cell surfaces (Watt and Ward, 1980).

Correlating the level of inhibition of adherence by the monoclonal antibodies with their effects on the negative surface charge of treated S. typhimurium SL/BR as assessed by pseudo-isoelectric point resulted in correlation coefficient of 0.78 for all the data. In contrast to the correlation of hydrophobicity with inhibition of adherence the correlation coefficients for IgA and non-IgA groups were also very good (0.72 and 0.93, respectively). There was also a less distinct clustering of isotypes than with the hydrophobicity correlation data.

It would appear that IgM and IgA both possess the ability to increase the negative surface charge of S. typhimurium SL/BR. The obvious role for this in preventing adherence to MDCK cell monolayers would be one of electrostatic repulsion. Both IgA and IgM are more negatively charged than IgG but they also both occur in polymeric form with the addition of J-chain. J-chain is only a small glucosamine-containing glycoprotein but it is extremely acidic in character due to an unusually high glutamic and aspartic acid content (Koshland, 1985). J-chain may aid both IgA and IgM in the prevention of

adherence by its highly negatively charged nature which would enhance the effect of electrostatic repulsion.

4.5 THE EFFECT OF TREATMENT WITH MOLECULAR FORMS OF IGA MONOCLONAL ANTIBODIES ON THE SURFACE PROPERTIES OF SALMONELLA TYPHIMURIUM SL3261 BRD026 (SL/BR) AND THE RELATION TO ADHERENCE ON MADIN DARBY CANINE KIDNEY (MDCK) CELL MONOLAYERS.

The multiple molecular forms of the two IgA Monoclonal antibodies (PIP18 and PIP2) all gave rise to inhibition of adherence comparable with that of the original unpurified maximal growth medium of their respective hybridomas. No significant difference was seen in the degree of inhibition produced by the molecular forms of the same monoclonal antibody.

Both the hydrophobicity, as determined by hydrophobic interaction chromatography (HIC) and the relative net negative surface charge of S. typhimurium SL/BR treated with these preparations was assessed. The results of the hydrophobicity effects were consistent with those achieved using their original maximal growth mediums for both the monoclonal antibodies (PIP18 and PIP2) with a decrease in the hydrophobicity of S. typhimurium SL/BR. The level of decrease was similar for all three preparations for both monoclonal antibodies and revealed no significant differences.

In contrast the molecular forms revealed significant differences when assessed for their effects on the

surface charge of S. typhimurium SL/BR. The 360 kD forms both gave an increase in the net negative surface charge comparable to that seen with the original maximal growth mediums. This increase was also seen with the 320 kD forms but was significantly lower ($p < 0.05$). The 160 kD forms had no significant effect on the surface charge of S. typhimurium SL/BR at all.

One possible explanation for the variation between the effects of the molecular forms on surface charge may lie in the physicochemical nature of J-chain, a protein associated with the dimeric form of IgA. J-chain is a highly negatively charged molecule (Koshland, 1985) and the possession or lack of this molecule in the three molecular forms may explain the changes in the surface charge of S. typhimurium SL/BR when treated with each form.

As already discussed, the difference in molecular weight between the 360 and 320 kD forms is probably caused by the lack or partial digestion of J-chain from the higher molecular weight form. If the high negative charge of J-chain is a primary cause of the increase in negative charge of S. typhimurium SL/BR treated with the 360 kD form then the lack of J-chain in the 320 kD form would explain the comparative lesser increase in negative charge of S. typhimurium SL/BR when treated with this form. The 160 kD preparation, monomeric in form, would not normally possess J-chain which is only found associated with the polymeric forms of IgA. Lack of J-chain would again explain the reduced increase in

negative charge when S. typhimurium SL/BR is treated with this form when compared with treatment with the 360 kD form.

Whether J-chain is the source of these differences the inhibition of these molecular forms can only be attributed to their effects on the surface hydrophobicity of S. typhimurium SL/BR and not surface charge as all give comparable inhibition but vary greatly in their effects on surface charge.

4.6 ASSESSMENT OF OPTIMIZATION OF CHEMILUMINESCENCE ASSAY PARAMETERS FOR THE COMPARISON OF IGA WITH IGM AND IGG1 MONOCLONAL ANTIBODIES.

Opsonization of Neisseria meningitidis organisms using human peripheral blood polymorphonuclear leucocytes (PMN) was measured by luminol-dependant chemiluminescence (CL) for 3 mouse monoclonal antibodies (IgG1, IgA and IgM) specific for type Y capsular polysaccharide. The CL profiles for each of these monoclonal antibodies were followed in order to compare their abilities as opsonic antibodies with relation to class.

Chemiluminescence profiles for each of the monoclonal antibodies were recorded in order to choose a time point at which a comparison could be made between the classes. The profiles of the 3 monoclonal antibodies differed in such a fashion that to choose any one time point for comparison would clearly be of little use.

In order to further understand the reason for the variation in their CL profiles a computer model was developed for the CL assay system. The computer model applied simple Michaelis-Menton kinetics to express the opsonization and subsequent CL and provided purely numerical solutions to the difference in profiles by assuming that those differences were caused by inequalities in the numbers of PMN available with class specific Fc-receptors for IgA, IgG1 and IgM.

The number of IgG1 antibody-bound bacteria was assumed to be acting as the limiting factor in the CL assay with the number of PMN expressing FC-receptors for IgG being in excess. With the IgA antibody, the number of PMN expressing FC-receptors for IgA was assumed to be acting as the limiting factor in the CL assay with the number of IgA antibody-bound bacteria being in excess. The system with IgM represented an intermediate stage between IgG and IgA.

This model was tested by altering the bacteria:PMN ratios for each of the Monoclonal antibodies to try to redress the inequalities by increasing the number of PMN available. The results indicated that with IgA the levels of PMN available for CL were not in excess, whilst for IgG1 the PMN were in excess.

The evidence for an inequality of PMN within peripheral blood which express Fc receptors for IgG compared with those for IgA is documented by various authors (Kilian et al., 1988; Fanger et al., 1983a, 1983b) . There is also evidence that this inequality is

reversed in areas close to mucosal surfaces (eg bronchial associated lymphoid tissue).

Fanger et al. (1983b) describe the increase in the number of macrophages expressing Fc-receptors for IgA in alveolar lavage collections compared with peripheral blood. They also found that incubation of peripheral blood neutrophils with IgA for 24 hours results in an increase in the numbers expressing Fc-receptors for IgA and the number of Fc-receptors for IgA per cell.

The use of traditional CL assays to compare IgG and IgA as opsonic antibodies may give a misleading picture of IgA as a poor opsonin due to a difference in the number of cells available to each class for Fc-receptor mediated CL.

4.7 CONCLUDING REMARKS.

The importance of mucosal immunity for the prevention of many serious diseases has been demonstrated by many workers (Besredka, 1927; Burrows and Haven, 1948; Arnold et al., 1978). The morbidity and mortality associated with enteric diseases in the third world identify this as an area in which the development of effective vaccines is a necessity (Levine et al., 1983). Current systemically administered vaccines are of limited efficacy and new vaccines, developed for oral administration, display the importance of inducing mucosal responses in order to prevent infection (Levine et al., 1983).

A primary aim of the project was the preparation of IgA secreting hybridomas by immunization of rats via Peyer's patches with subsequent use of mucosally derived lymphocytes for fusion.

The work admirably demonstrates that this protocol is an extremely efficient and successful method for generating IgA hybridomas of various monomeric and polymeric forms.

The monoclonal antibodies generated were directed against a number of antigens of S. typhimurium, with the majority specific for the immunodominant O-antigen of LPS.

These monoclonal antibodies were to be used for in vitro analysis of IgA function in preventing adherence of S. typhimurium to epithelial cells and a comparison of this effect with monoclonal antibodies of other isotypes prepared in parallel using the same protocol.

In order to do so, a novel in vitro assay system for the measurement of adherence of S. typhimurium to Madin Darby canine kidney (MDCK) epithelial cell monolayers was devised and standardized with which to study selected anti-LPS monoclonal antibodies of IgA, in comparison to monoclonal antibodies of IgM and IgG1 isotype.

The success of this assay system in demonstrating both the adherence of S. typhimurium SL/BR to MDCK cells and the ability of monoclonal antibodies to inhibit this adherence should allow further work to take place using this system in the investigation of the effects of other immune molecules (eg lactoferrin) on the adherence of S.

typhimurium in vivo. The system also allows for further investigation of intracellular transport (transcytosis) of S. typhimurium with the fully polarized growth of MDCK monolayers on permeable supports.

The comparison of isotypes in the adherence assay was complicated by the presence of only one IgG monoclonal antibody, but as was seen comparison of the opsonic capabilities of isotypes also suffered problems.

The assessment of the opsonic effects of varying isotypes using a conventional luminol-dependant chemiluminescence assay under normal conditions gave misleading results. The use of human peripheral blood polymorphonuclear leucocytes (PMN) gave rise to a bias against IgA due to an inequality in the presence of PMN with Fc-alpha as compared to Fc-gamma receptors. Such results should illustrate the problems involved in comparing IgA to other isotypes in conventional systems derived from systemic rather than mucosal studies.

In contrast, the adherence assay used in this study allowed the comparison of IgA monoclonal antibodies with other isotypes from a "mucosal" standpoint.

The most striking results were seen when comparing the varied physico-chemical effects of monoclonal antibodies of differing isotype on the surface properties of S. typhimurium SL/BR. The effect on surface properties appeared to be related to isotype rather than specificity, with each isotype giving characteristic changes.

In general, changes in the surface hydrophobicity of S. typhimurium SL/BR did not correlate with the inhibitory effects of the monoclonal antibodies, whereas increases in negative surface charge did. Both IgA and IgM monoclonal antibodies gave increases in surface charge whilst inhibiting adherence. The IgG1 antibody which gave no increase in surface charge was the only monoclonal antibody tested which did not inhibit adherence.

From these results it appears that the effects of IgA and IgM in preventing adherence at the mucosal surface may rely as much on the non-specific physicochemical effects related to isotype as to specificity for, and blocking of, specific adhesins. The importance of these non-specific physicochemical effects of IgA and IgM at the mucosal surface may increase when compared with the results of the one IgG1 antibody. With only one antibody no firm conclusions can be reached, however it's physicochemical properties and it's inability to prevent adherence may parallel situations in vivo where pathological effects on mucosal surfaces are seen in conjunction with a switch in isotype from IgA to IgG. Whilst specific antibody continues to be produced the non-specific physicochemical effects related to isotype may impair clearance of microorganisms and lead to resultant pathological consequences.

Although this is only speculation, the results of this project would indicate that the physicochemical

nature of isotypes at the mucosal surface, particularly IgA, are of some importance and warrant further study.

4.8 CONCLUSION.

In conclusion, IgA and IgM Mabs directed against LPS were found to inhibit adherence of *S. typhimurium* to MDCK cell monolayers whereas the one IgG1 anti-LPS antibody did not. Treatment of the bacteria with these Mabs had variable effects on bacterial hydrophobicity but this did not correlate with their ability to inhibit adherence. In contrast all inhibiting Mabs increased bacterial net negative surface charge, with the level of increase correlating well with the degree of inhibition of adherence. This would appear to show the importance of the effect of surface charge on bacterial adherence and to posit a mechanism for antibody interference based on physicochemical alteration of bacterial surface charge. Although the results of the charge effects of IgA molecular forms did not seem to support this idea it is likely that the effect of IgA on reducing bacterial hydrophobicity could be responsible for the inhibition seen with the monomeric forms in the absence of any effect on bacterial surface charge. In fact this lack of effect on surface charge may support a possible role for J-chain, a highly negative charged molecule associated with both polymeric IgA and IgM, as responsible for the charge effects seen with both IgA and IgM but not IgG1. Though surface charge effects may not fully explain all bacterial-cell interactions this work presents strong evidence for a major role for the surface charge related effects of secretory antibodies (IgA and IgM) on bacteria and their inhibitory effect on bacterial adherence.

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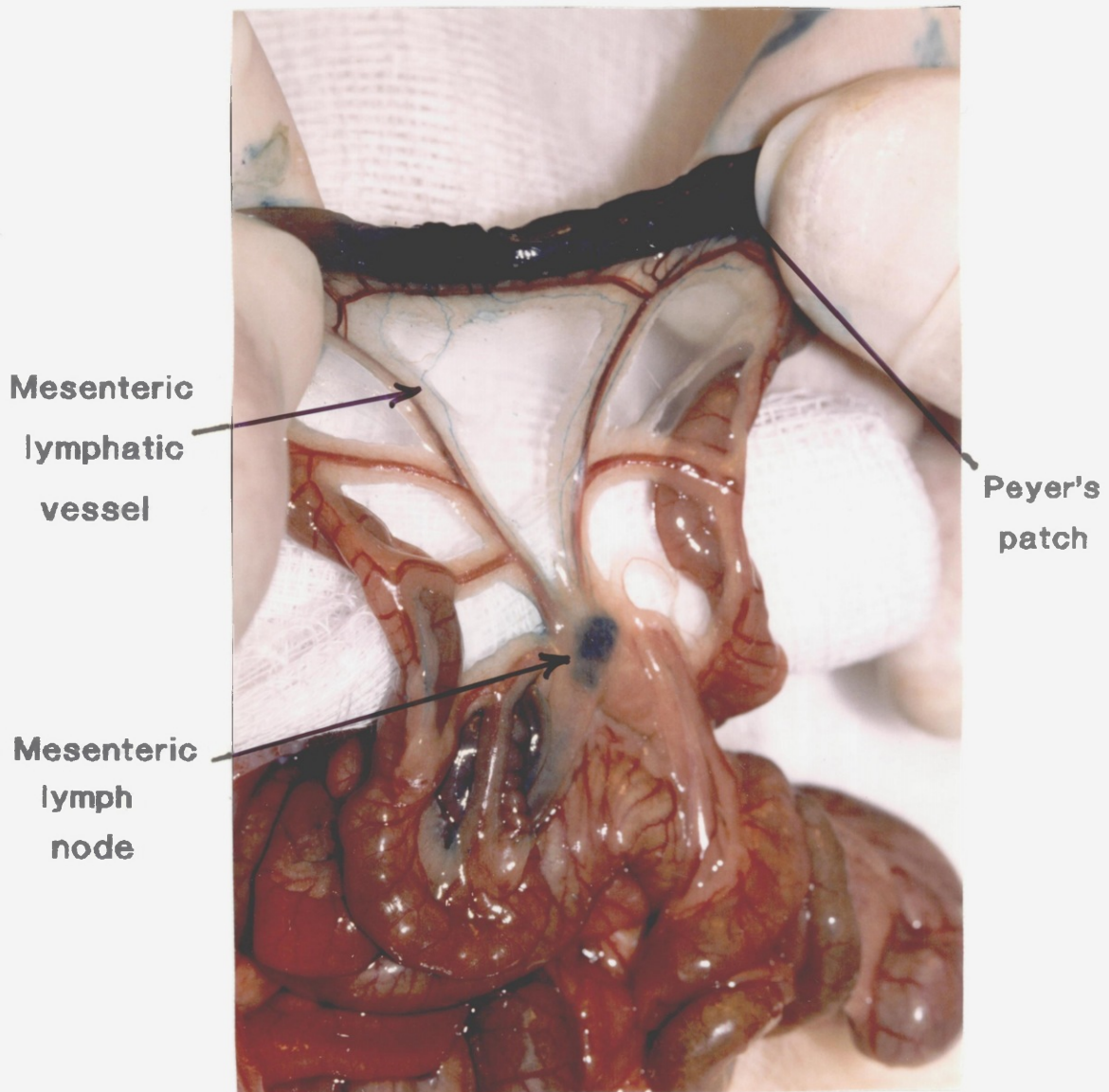
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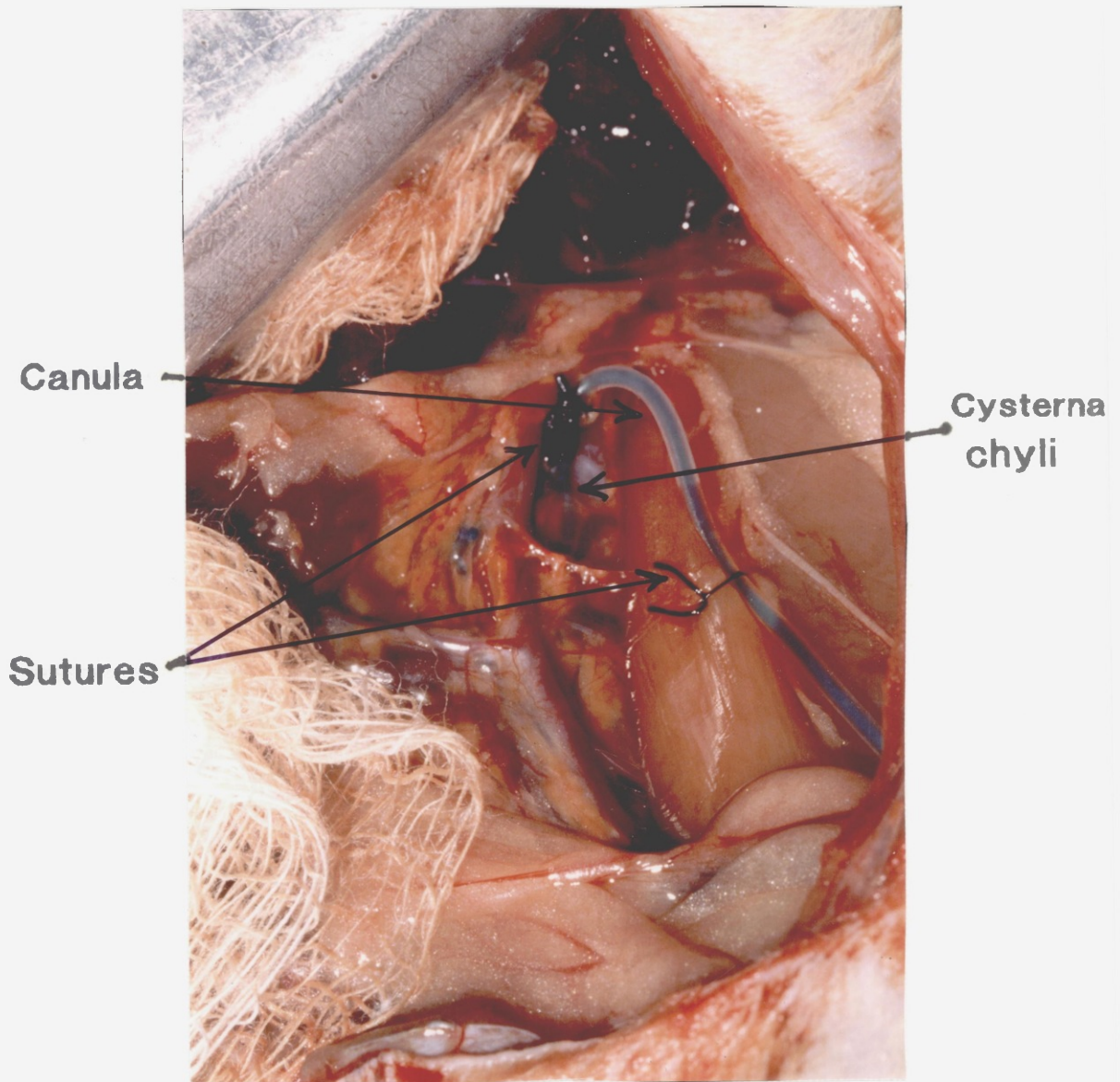
6.APPENDIX

Figure 29. Photograph of rat mesenteric lymphatic system, showing mesenteric lymph node, mesenteric lymphatic vessels and Peyer's patches.



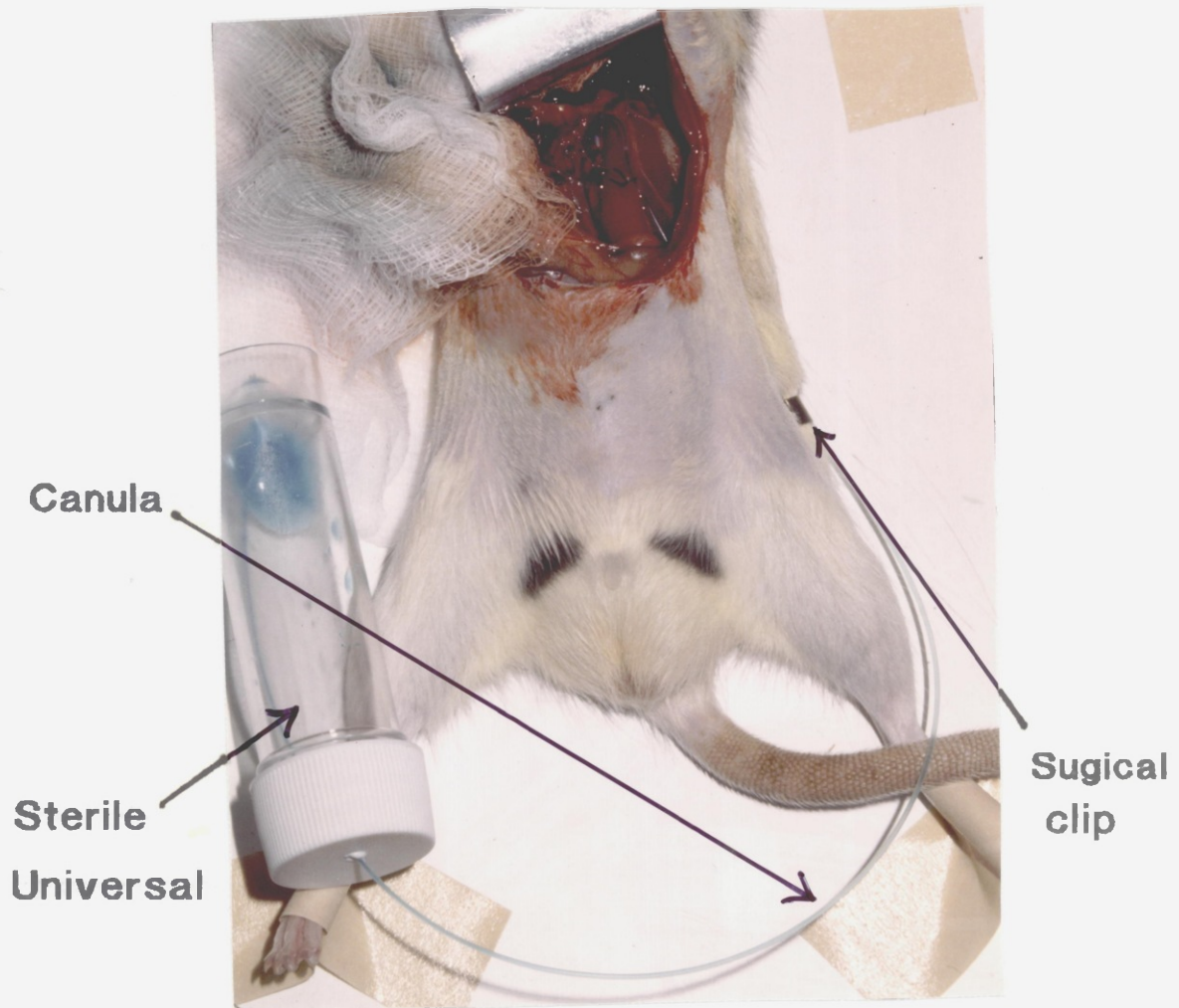
Note: The visualisation of the lymphatic vessels and organs has been aided by the injection of a small amount of lymphography dye (Guerbert's Patent Blue) into a number of Peyer's patches. Under normal conditions the vessels are clear and translucent. Peyer's patches may be more easily seen by gently squeezing the patch between the fingers.

Figure 30. Close-up photograph of rat abdomen showing the
canulation of the cysterna chyli.



Note: A lymphography dye was used as in Figure 29 to aid visualisation. The canula can be clearly seen and the placement of the sutures amongst the surrounding musculature is such that once the abdomen is closed the canula is held securely.

Figure 31. Photograph of rat showing canulation of cysterna chyli with canula passing through the abdominal wall into a sterile universal.



Note: The canula is passed through the abdominal wall close to the hind leg of the rat in such a way that it can be secured by a surgical clip.