PRODUCTION AND CHARACTERISATION OF MONOCLONAL ANTIBODIES TO *VIBRIO CHOLERAE*

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ABBREVIATIONS

Abbreviations used are as recommended in the Biochemical Journal Instructions to Authors, 1981, with the following additions:

FCS	Foetal calf serum
PEG	Polyethylene glycol
BSA	Bovine serum albumin
ELISA	Enzyme linked immunosorbent assay
IF	Immunofluorescence assay
LPS	Lipopolysaccharide
Ig	Immunoglobulin
MCAb	Monoclonal Antibody
SDS-PAGE	Sodium dodecylsulphate-polyacrylamide gel electrophoresis

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SUMMARY

Ogawa and Inaba are the two main serotypes of V. cholerae 01, the etiologic agent of the severe diarrhoeal disease, cholera. In the gut of human hosts, the vibrios produce a cholera toxin which causes rapid dehydration. Various field and volunteer studies (Levine et al, 1979, 1981; Cash et al, 1974) have indicated that antibacterial activity is more important than antitoxin activity in protection against subsequent challenge infection.

V. cholerae antigens have been classified by conventional serology into three groups. The A antigen is common to Inaba and Ogawa, the B is specific to Ogawa and the C specific to Inaba.

The structural analysis of V. cholerae LPS has received very limited attention and hence the antigens responsible for homologous and heterologous vibriocidal activity are not well understood. Recent chemical analyses of the V. cholerae LPS have, however, suggested that the group antigen is a repeat of perosaminyl residues whose amino functions are acylated by L-glycero-tetronic acid (Redmond, 1979; Kenne et al, 1982). However, the type specific antigens of Ogawa and Inaba are still obscure.

The objective of the present study was to investigate the shared and unique epitopes of Ogawa and Inaba serotypes with MCAbs. A panel of six MCAbs was produced and assessed for specificity to the two serotypes and other potentially cross reacting bacteria. All were directed primarily to the LPS rather than membrane protein. The specificity profile of each antibody was heavily dependent on the assay system employed and the antibodies could be classified into three groups. Three could be demonstrated to be totally specific to Inaba LPS on some assay systems but to show extensive cross reaction with Ogawa vibrios on other assay systems. Two antibodies were totally specific to Ogawa vibrios on all assay systems. A sixth antibody could be shown to be totally specific on Ogawa vibrios in some assay systems and to be totally specific to Inaba on other assay systems. The dual specificity of the four cross reactive antibodies could be related to the use of washing as opposed to nonwashing assays, the concentration and method of presentation of antigen and antibody, and the density, accessibility and geometry of the antigen on the bacterial surface.

These results indicate that the conventional classification of V. cholerae antigens does not take into account more subtle interactions between antigen and antibody. In addition, they emphasise the point that emerging hybridomas must be screened under the exact conditions of final use of the antibody to be generated. Finally, they indicate that a single antibody may be employed in a dual capacity, detecting different serotypes in two different assay systems.

The interaction of the antibodies with the vibrio LPS molecules meant that it was necessary to resolve these molecules in order to analyse the interaction. High resolution electrophoresis was necessary to do this as the size of the repeating unit was discovered to be exceedingly small (less than 300 daltons) compared to that observed for Salmonella typhimurium and E. coli O111:B4. Most interestingly, no differences in the mobility, number of side chains or the density of the O-chains were detected on SDS-PAGE for the two serotypes. Thus, the difference in serotype cannot be related to difference in electrophoretic mobility even at this fine resolution. Nonetheless, the MCAbs were able to make clear distinctions between the two serotypes on immunoblotting, emphasising their considerable power as a specific detection system. The results suggest that the difference between Ogawa and Inaba must therefore be interpreted in terms of LPS molecules which have O-side chains with very small repeating units identical in size and electrophoretic mobility but containing enough structural differences to allow them to react differentially with serotype specific monoclonal antibodies.

INTRODUCTIION

1.1 General Introduction

Cholera-like diseases have been common on the Indian subcontinent for many centuries and are reported in some of the earliest medical records. Susrata-samhita, a treatise on Indian medicine, written around the seventh century A.D. has descriptions of illness and death due to dehydrating diarrhoea and vomiting. It is likely that no long-distance spread of cholera took place before the nineteenth century despite clear descriptions of epidemics on the Indian subcontinent from the late fifteenth century onwards in the journals of Portuguese explorers. The first of the seven cholera pandemics erupted from India in 1817 but did not travel very far into Western countries. The second pandemic reached Moscow, Great Britain and America killing thousands of people between 1831-1849. The fifth pandemic in 1881 was studied by the French and German cholera commissions under Isadore Straus and Robert Koch in Egypt and later by Koch in Calcutta. Koch identified *V. cholerae* as the causative agent of cholera and in 1887, proposed that the disease was toxin mediated. It was only after De (1959) and Dutta (1959) reported their studies from India that the existence of cholera

toxin was finally established. De (1959) injected cholera culture filtrate into ligated loops of rabbit small intestine and showed that a few hours after the injection, the loops swelled up with rice-water fluid characteristic of cholera stools. Ten years later, methods for the preparation and purification of cholera toxin were established by Finkelstein and Lo Spalluto (1969) and its effect on the adenylate cyclase-cyclic AMP system was soon established. Since then, activation of adenylate cyclase by cholera toxin has been shown to occur in most mammalian cell types, the stucture-function relationship of the toxin has been defined, the cell receptor identified and most recently, the mode of action of the toxin on adenylate cyclase explained in considerable detail. Studies on V. cholera, the etiological agent of cholera, however, have lagged behind.

1.2 Epidemiology of cholera

Epidemic and pandemic cholera is caused by V. cholerae 01 of which two biotypes exist, classical and El Tor. The first El Tor vibrios were isolated by Gotschlich in 1905 at the El Tor quarantine station in the Sinai peninsula. The vibrios isolated were agglutinated with cholera antiserum and resembled cholera vibrios described earlier by Koch but the organisms produced haemolysin which was not observed with the classical strain of cholera. It was not until 1961 when the El Tor variant produced an epidemic of major proportions in the Philippines that there was general agreement that the haemolytic V. cholerae could be responsible for severe epidemic human disease. Between early

1960s and 1980 the El Tor biotype spread throughout the world to the virtual exclusion of classical biotype organisms. However, in 1982 diarrhoea due to the classical biotype reappeared in epidemic form in Bangladesh (Samadi et al, 1983) and it remains to be seen whether classical cholera will again predominate in the Gangetic delta and whether it will spread elsewhere in Asia or Africa as the seventh pandemic is still with us.

1.2.1 Toxin genes of classical and El Tor biotypes

The analysis of cholera toxin gene stucture has shown that V. cholerae strains of the classical biotype contain a nontandem, chromosomal duplication of the ctx operon that is structurally identical in all strains (Mekalanos et al, 1983). In contrast, about 70% of El Tor strains have only a single copy of ctx, while the remaining strains have two or more ctx copies present on a tandemly repeated genetic element. This duplication and amplification of the toxin operon may be related to the virulence of the serotypes.

1.2.2 Association of cholera with ABO blood group

Many diseases are associated with ABO blood group classification. Most of them occur in adults after their reproductive age and are not fatal, so they have been considered to be of little evolutionary consequence (Mourant and Kopec, 1978). Associations of ABO grouping with susceptibiltiy to major epidemic diseases such as plague and smallpox might account for greater genetic selection but these suggested relationships have

only been based upon known or supported molecular similarities between the etiological agent and blood group antigens and have not been identified in population-based studies (Springer, 1971). Barua and Paguio (1977) and Chaudhuri, 1977 observed that in endemic areas patients hospitalised with cholera were more likely to be of blood group O than the control population. Levine et al, (1979) had also reported that US volunteers of O blood group who were challenged with V. cholerae 01 tended to have more severe purging than non-O group volunteers.

Glass et al, (1985) examined the blood groups of patients hospitalised for diarrhoeal disease due to a variety of bacterial and viral agents. The authors identified a significant association only with the disease cholera, in which cholera patients were twice as likely to have blood group AB as community controls. Family contacts of cholera patients indicated that blood group did not affect an individual's risk of having a culture-proven infection with V. cholerae 01 but was directly related to the severity of disease. Individuals with the most severe diarrhoea were more often of blood group O and less often of AB compared with those with asymptomatic infection. Patients infected with enterotoxigenic E. coli, which expresses a heat-labile toxin similar to cholera toxin, did not show a similar distribution of blood group antigens. In addition, O blood group patients were at no increased risk of infection with V. cholerae non 01 which is genetically related to V. cholerae 01 but does not normally produce cholera toxin.

While the authors were not able to identify the molecular

basis for this apparently genetically related protection, they have suggested that the constant selective pressure of cholera against people of O blood group may account in part for the extremely low prevalence of O group genes and the high prevalence of B group genes found among the people living in the Gangetic Delta.

1.3 Morphological characteristics of V. cholerae

V. cholera is a gram-negative bacterium. The bacteria are short and curved and have a single polar flagellum. Motility provided by the vibrio's unipolar flagellum is important for its virulence (Schrank and Verwey, 1976).

1.4 Cholera Infection

Humans are the only known natural hosts of V. cholerae 01 infection and in nature they usually ingest these organisms by means of contaminated water (Levine and Nalin, 1976) or food, typically sea food (Dutta et al, 1971). The probable inoculum size in nature is believed to be 10^3 to 10^6 organisms and this is corroborated by volunteer studies in which ingestion of 10^6 classical Ogawa 395 organisms with 2.0g of sodium bicarbonate by North American volunteers resulted in diarrhoeal illness (Levine et al, 1979). Gastric acid in normochlorhydric persons serves as a very competent nonspecific defence barrier (Levine et al, 1981).

When V. cholerae 01 organisms successfully pass through the pylorus in viable state, they reach the critical anatomic

site of host-bacterium interaction, the proximal intestine. Here a complex series of events occurs as the vibrio, using an array of virulence mechanisms, attempts to overcome a number of nonspecific defence systems employed by the host. Two nonspecific defence mechanisms work in unison, the small intestine peristalsis and a mucus layer coating the small intestine. V. cholerae organisms penetrate the mucus layer and rapidly reach the enterocytes of the small intestine to which they attach themselves. In this manner they overcome the normal defences and colonize the small intestinal mucosa (Levine et al, 1983) and elaborate the enterotoxin, cholera toxin. The vibrios can secrete a potent mucinase which is believed to assist their entrance into the mucus gel covering the mucosa (Lam et al, 1955).

1.5 Cholera toxin and its mode of action

Like many other bacterial diseases, cholera may range from a symptomless infection to a severe attack rapidly proving fatal. In its most characteristic clinical form it starts abruptly with diarrhoea, soon marked by the passage of rice-water stools and precipitating vomiting. Several litres of body fluid may be lost within a few hours which leads to shock and death if the fluids are not replaced. The diarrhoea is caused by the bacterial toxin rather than by a direct action of the bacteria themselves.

Cholera toxin is a protein of M_r 87K which consists of an A_1 peptide linked by a disulphide bond to an A_2 peptide and five B peptides. The B subunits are responsible for cell binding

and the A subunits for the direct toxin activity.

The first event in the action of cholera toxin on cells is the rapid and tight binding to receptors on the cell surface. The membrane receptor for cholera toxin is a G_{M1} ganglioside (Holmgren et al, 1973; King and Heyningen, 1973; Cuatrecasas, 1973). This carbohydrate-rich sphingolipid is recognised by the B chains of the toxin. The cell penetration by the toxin is as yet poorly understood. After gaining entry into the cell the A_1 subunit catalyses the transfer of an ADP-ribose unit from NAD^+ to the guanosine nucleotide-binding component of the membrane bound adenylate cyclase (Moss et al, 1976). Adenylate cyclase is active while GTP is bound to the GTP-binding component but reverts to an inactive state as GTP is hydrolysed to GDP by GTPase (Cassel and Selinger, 1977). Cholera toxin blocks the GTPase action thus stabilising the active conformation of adenylate cyclase. Altenatively, cholera toxin may stimulate adenylate cyclase by enhancing an exchange reaction in which stimulatory GTP replaces inhibitory GDP at a rate higher than that of hydrolysis of GTP to GDP (Lad et al, 1980). The increase in the adenylate cyclase activity of the mucosa of the small intestine raises the level of cAMP in these cells. The abnormally high level of cAMP stimulates active transport of ions by the intestinal epithelial cells, which results in a large efflux of Na^+ and water into the gut.

1.6 Prevention and therapy of cholera

1.6.1 Rehydration therapy

Bacteriophage or serum treatment have not proved to be of any therapeutic value but chemotherapy provides an useful complement to the main purpose of treatment, which is the replacement of the lost fluid and electrolytes (Chaudhuri, 1971; Pierce et al, 1970). The amount and composition of the blood plasma has to be restored as rapidly as possible by a suitable fluid which contains almost exactly the electrolyte content of the stools. During the past decade, oral rehydration therapy by means of appropriate glucose-electrolyte solutions has simplified the treatment of dehydrating diarrhoea, making effective therapy feasible in places where intravenous treatment facilities are limited or unavailable. This treatment takes advantage of the presence of an uptake mechanism for sodium in conjunction with certain organic solutes, including glucose, which is not regulated by cyclic nucleotides and thus is unaffected in cholera and other forms of enterotoxic diarrhoea. However, in severely affected patients purging often occurs at such a high rate that balance cannot be maintained by oral fluid only and intravenous replacement of fluid is essential for survival.

1.6.2 Prevention of the disease

Cholera can be controlled by good sanitation and personal hygiene. The preventive measures required are similar to those taken against other infections of the enteric type, except that

no provision is needed against chronic carriers because these rarely occur in cholera. Clean water supplies and safe disposal of human sewage can dramatically reduce the number of clinical cases of cholera.

1.7. Immunity against cholera

1.7.1 Immunity following infection

In endemic areas the incidence of cholera is highest in children 2 to 4 years of age and incidence rates diminish thereafter with increasing age (Glass et al,1982). At the same time the prevalence of vibriocidal antibody in serum increases with age (Mosely et al, 1973; Mosely et al, 1968). These epidemiological observations point to the development of acquired immunity from clinical and subclinical infection. On the other hand, repeated infections in endemic areas, usually not attended by clinical symptoms, have been reported. Genuine second attacks occurring within a few weeks of the first were observed by Woodward (1971).

The majority of the analyses on the quality and duration of infection-derived immunity have been conducted on healthy adult volunteers (Clements et al, 1982; Levine et al, 1981), who were not very representative of the undernourished population of the third world, the centre of endemic areas. However, such studies do throw some light on the knowledge of acquired immunity. Cash et al, (1974) showed that an initial clinical

cholera infection due to classical Inaba vibrios confers immunity to rechallenge with the homologous organism. Levine et al, (1979) subsequently showed that an initial clinical infection due to classical vibrios of either serotype led to complete protection upon subsequent challenge with classical vibrios of the heterologous as well as the homologous serotypes. Not only were the rechallenged volunteers clinically protected but also classical vibrios were never recovered by direct culture of stools. The immunity was also shown to last for three years, the longest interval tested.

Levine et al, (1981) also undertook to examine the immunity conferred by clinical infection with El Tor vibrios. An initial infection due to V. cholerae El Tor was found to provide significant protection against rechallenge with El Tor vibrios of either homologous or heterologous serotype. In contrast to the experience with classical vibrios in volunteers, individuals rechallenged with El Tor vibrios often had positive direct coprocultures. It was not established whether this was due to an enhanced capacity of El Tor vibrios to survive in nondiarrhoeal stool or whether El Tor vibrios stimulate less potent intestinal immunity than the classical vibrios.

1.7.2 Antigen-stimulated immunity in humans

Multiple antigens are elaborated by V. cholerae, among them are lipopolysaccharides (LPS), flagellar sheath proteins, various haemagglutinins and outer membrane proteins. Each of these antigens may play a critical role in pathogenesis.

Presumably, the host immune responses directed against these antigens serve as mediators of the potent immunity observed in volunteer studies. Information on the immune response is crucial to vaccine development to help determine what antigens are particularly desirable for inclusion in immunising agents.

1.7.2.1 Anti-LPS antibodies

After cholera infection, serum and intestinal or milk secretory immunoglobulin A (SIg A) antibody responses can be demonstrated by ELISA against purified LPS antigen (Majumdar and Ghose, 1981; Majumdar et al, 1981). Serum vibriocidal antibody assayed against live V. cholerae in the presence of complement is directed mainly, but not entirely, against the LPS antigen. Serum vibriocidal antibody levels rose significantly in more than 90% of North American volunteers who participated in cholera challenges (Clements et al, 1982).

1.7.2.2 Antibodies to flagellar sheath protein

There have been no reports to date of the serum or local intestinal antibody response to flagellar sheath protein in man.

1.7.2.3 Antibodies against cholera haemagglutinins

The detection of human response against cholera haemagglutinins has also not been reported to date.

1.7.2.4 Expression of outer membrane protein directed antibodies

Sears et al, (1984) have characterised the frequency, magnitude and kinetics of the serum antibody response of humans to the major outer membrane proteins of V. cholerae after experimental cholera infection in challenge volunteers. 50% of 79 volunteers challenged with the El Tor biotype and 54% of 35 volunteers challenged with the classical biotype had significantly increased levels of IgG anti-outer membrane protein antibodies. Sera from paired volunteers showed significant rise of antibody as measured on ELISA when tested against outer membrane protein of either serotype. However, vibriocidal activity of the anti-outer membrane protein antibodies was not tested. Absorption of antisera with LPS resulted in only 12% decrease in anti-outer membrane protein activity on ELISA.

1.7.2.5 Anti-toxin antibodies

Cholera toxin is a potent antigen, the resultant immune response being directed entirely against the B subunit (Holmgren et al, 1981; Peterson et al, 1979). All neutralizing activity of cholera antitoxin can be absorbed with a purified B subunit.

More than 90% of North American volunteers who ingested V. cholerae while serving as controls in challenge studies to assess vaccine efficiency developed rises in serum antitoxin which was measurable in either toxin-neutralizing assays or as IgG on ELISA (Robins-Browne et al,1980). Serum IgG antitoxin is long lived, elevated levels being demonstratable in most North

Americans at least two years after experimental cholera infection (Levine et al, 1977).

Approximately 60% of volunteers who participated in experimental cholera studies manifested increased levels of SIgA antitoxin in intestinal fluid measured before and 9 days after challenge (Levine et al, 1981). The local antitoxin response tends to be relatively short-lived, with antibody levels usually dropping by one month post challenge. Levine et al, (1979) demonstrated that purified cholera toxoid is antigenic in man when given enterally or orally, but fails to provide protection against experimental challenge.

1.8 Local immune defences

Since cholera is entirely a topical infection it would seem most likely that topical defences would be the main determinant of protection against infection by V. cholerae. Recurrent infections are rare in cholera and the incidence of disease decreases rapidly with age (Gangarosa and Mosely, 1975). Thus an effective immunity does seem to occur in cholera. This has been further documented by volunteer studies in which subjects who had previously been challenged with V. cholerae and had contracted the classical illness were rechallenged after a period of 3-6 months. Such people proved highly resistant to rechallenge despite rather low circulating antibody titres (Cash et al, 1974). Early work recognising local antibody in contrast to serum antibody as a potentially important protective defence against experimental cholera has been reviewed (Pollitzer,

1959). From this awareness has evolved a more detailed knowledge of how local immune mechanisms in the intestine function. Antigens are detected by Peyer's patch lymphocytes. These immature cells then migrate through the lymphatics to the circulation and are 'processed' at a site that has not been fully clarified as yet. The 'processed' lymphocytes then return to the gut, and perhaps to other tissues and tend to locate themselves in areas where antigen is present. Their product once back in the gut mucosa is secretory IgA. Studies using cholera toxin as a probe have led to a description of this immunocyte traffic as it pertains to cholera (Pierce and Gowans, 1975).

The question of how local secretion of IgA or the presence by exudation from the serum of IgG or IgM can mediate destruction of vibrios in an environment where complement does not function has challenged the ingenuity of investigators. There is no such problem with respect to how antitoxic antibodies may work. If there is a layer of antibodies adjacent to the epithelium that will bind and deactivate toxin before it attaches to the cells, this will prevent all the disease manifestations. Thus in the surface of the intestine such antibodies may play an important role in defence against clinical illness if not against the infection itself. It is possible that failure by the upper intestine to secrete fluid in response to toxin limits the growth medium available for the multiplication of V. cholerae and so in fact antitoxin antibodies may at least limit the numbers of vibrios in an important way (Levine et al, 1979). Several mechanisms by which the growth of V. cholerae can be inhibited

are likely to operate within the host. The process by which the vibrios attach themselves to the gut epithelium is highly specific (Jones and Freter, 1976) and antibodies against whole vibrios interfere with this process. Motility is important in the pathogenesis and antibodies against whole vibrios or specific O-antigen will cause clumping and arrested motion. It is unlikely that phagocytosis or vibriolysis is important in the gut.

1.9 Vaccines against cholera

The various forms of vaccines that have been tested against cholera were reviewed by Levine et al, (1983) and their relative efficiency is discussed below.

1.9.1 Toxoid vaccines

These are immunising agents intended to prevent cholera by means of stimulating antitoxic immunity. Various forms of the vaccine have been tried. They are, formaldehyde treated cholera toxin, glutaraldehyde treated cholera toxin, the purified B subunit and the procholeragenoid.

Toxoid vaccines stimulate purely antitoxic immunity and have not been shown to be efficacious in protecting humans against cholera (Levine et al, 1979), although they may protect animal models (Fujita and Finkelstein, 1972; Holmgren and Svennerholm, 1977; Pierce et al, 1983).

1.9.2 Killed whole cell vaccines

1.9.2.1 Parenteral whole-cell vaccines

In field trials, parenteral killed whole-cell vaccines have been shown to confer significant protection against the homologous *V. cholerae* serotype, usually for a period of less than one year. There is some evidence to suggest that parenteral whole-cell Inaba vaccine provides good short-term protection against Ogawa as well as Inaba cholera (Mosley *et al*, 1973) where as Ogawa vaccine is effective only against Ogawa (Phillipines Cholera Committee, 1973). The whole cell vaccine probably fails because it lacks any toxin-derived antigen and because the injection route may be relatively inefficient in stimulating local immunity in the gut mucosa (Pierce, 1978).

1.9.2.2 Oral whole cell vaccines

Killed vibrios have also been used as oral vaccines and have been shown to stimulate the appearance of local intestinal antivibrio antibody (Freter and Gangarosa, 1963; Ganguly *et al*, 1975), for a short period of time.

1.9.3 Combination vaccines

Combinations of antigens such as toxin plus LPS that stimulate both antitoxic and antibacterial immunity give synergistic protection over that provided by each antigen alone (Holmgren and Svennerholm, 1977). However, the protective vaccine has only moderate efficacy, 27-67% and multiple doses

are required to induce the protection.

1.9.4 Attenuated V. cholerae vaccines

The observation that both classical and El Tor clinical cholera infections in North American volunteers stimulated a high degree of protective immunity for at least 3 years (Cash et al, 1974; Levine et al, 1981) intensified research on the development of new cholera vaccines. As a result of these observations, the most promising approach would seem to be toward immunological control of cholera by means of attenuated non-enterotoxigenic V. cholerae strains used as oral vaccines.

1.9.4.1 Chemically mutagenised attenuated strains

Honda and Finkelstein (1979) mutagenised El Tor Ogawa 3083 with nitrosguanidine and produced Texas Star-SR strain. This strain produces normal or increased amounts of B subunit but is negative in assays for holotoxin activity or A subunit activity. Texas Star-SR strain has been evaluated as a live oral vaccine in humans (Levine et al, 1984). The clinical studies demonstrated that mild diarrhoea occurred in one-fourth of the recipients of Texas Star-SR, although it does not elaborate holotoxin. The strain was able to colonise the duodenum and stimulate local and circulating antibody responses. Finally, one or two doses of vaccine provided a moderate degree of protection (61% vaccine efficiency) against experimental challenge with high inocula of V. cholerae El Tor of either serotype which caused cholera in 70-80% of control volunteers. However, the Texas Star strain

itself suffers from certain drawbacks. Firstly, the method of attenuation, mutagenesis with nitrosguanidine, induces multiple mutations, not all of which are recognised. Secondly, the precise lesion presumed to be responsible for the attenuation of Texas Star is not known and consequently, until this is clarified there always remains the theoretical possibility of reversion to virulence.

1.9.4.2 Genetically engineered mutants

Recombinant DNA techniques have recently been applied to develop attenuated V. cholerae oral vaccine with none of the above mentioned (Section 1.9.4.1) drawbacks (Kaper and Levine, 1981; Mekalanos et al,1983; Kaper et al, 1983). Precise deletions of the genes encoding the cholera toxin or the A subunit have been achieved in strains whose pathogenicity and ability to confer protection were demonstrated in volunteer studies. These recombinant DNA techniques can predictably and repeatedly yield non-enterotoxigenic variants for use as potential live oral vaccines. Field studies have to be conducted to evaluate the safety and efficacy of vaccine candidates produced by this method.

1.10 The antigenic structure of V. cholerae

Gardner and Venkatraman (1935) subdivided vibrios into six groups, each with a different O antigen. The O1 antigen, the specific antigen of the cholera vibrio is not shared by any other serotypes. All the serotypes of V. cholerae share a heat labile

flagellar H antigen. An H antiserum can agglutinate all motile strains of the cholera group. It is possible that more than one H antigen exists although, the major H antigen is common to all members of the cholera group of vibrios.

The O antigen is a heat stable antigen. Kauffmann (1950) examined the classification on the basis of agglutinaton and agglutinin absorption tests and concluded that the cholera vibrios of Gardner and Venkatraman O group 1 are Inaba and Ogawa. Inaba vibrios are with antigens A and C and Ogawa or Ogawa-Hikojima with antigens A, B and (C). Formerly, Ogawa strains had been regarded as having A and B antigens and Hikojima strains as having A, B and C antigens but Kauffmann observed that Ogawa strains grown at 20^{0}C, could totally absorb anti-Inaba sera, as could Hikojima strains and the difference between Ogawa and Hikojima forms appeared to be largely of a phenotypic and quantitative nature. In practice he noticed that the antigens of Inaba could be reduced to A and for Ogawa to B. Sakazaki and Tamura (1971) confirmed and extended these findings and concluded that the differences between the Ogawa and Hikojima forms were largely quatitative and dependent on the proportion of B and C antigens present in addition to the common A antigen. They also suggested that the Inaba form was derived from Ogawa by the loss of somatic antigen B. Inaba, Hikojima and rough variants were readily recovered from Ogawa strains. El Tor serotypes Inaba, Ogawa and Hikojima are serologically identical to the respective serotypes of classical biotype.

Serotypic conversions also occur in nature. It has

frequently been observed that the heterologous serotype may be isolated, usually late, in the course of an outbreak originally caused by the other serotype (Finkelstein,1973). Samadi et al, (1983) have suggested that there may be more crucial biological characteristics other than the taxonomic traits used to identify the classical and El Tor strains, which gives new strains an advantage over the existing ones.

The current classification of such vibrios is therefore that the Ogawa serotype has antigens A and B and the Inaba has antigens A and C with Hikojima, should it exist having all three.

1.11 Reference sera for identification of V. cholerae serotypes

Sera raised against V. cholerae 01 represents the group serum and by the use of absorbed sera, the cholera vibrios are divided into serotypes. By absorption of 01 antiserum with Ogawa organisms a serum is prepared that agglutinates Inaba but not Ogawa. Similarly if the 01 antiserum is absorbed with Inaba organisms, the serum agglutinates Ogawa organisms but not Inaba. The recognition of these serotypes depends on the quality of the sera and the completeness of the absorption.

1.12 The chemical nature of somatic antigens

Watanabe and Verwey (1965) and Watanabe et al, (1965) reported the isolation of a biochemically and immunologically homogenous, large (100S) LPS antigen, from culture supernatants of an Ogawa serotype El Tor vibrio, and shown this to be highly

protective in mice against intraperitoneal challenge with live vibrios suspended in mucin. Antisera produced against the antigen were mouse protective, vibriocidal and precipitating but had little agglutinating activity although the antigen absorbed all these activities from sera prepared with whole bacterial cells. There was essentially no cross-reaction or cross-reactivity with Inaba vibrios or antigens. The antigen was low in nitrogen content and was approximately 60% carbohydrate. The antigen was isolated by ammonium sulphate precipitation followed by deproteinisation by phenol extraction, lipid removal with chloroform-methanol and controlled cold ethanol fractionation.

A mouse protective antigen was also isolated from an Inaba serotype by the same group (Verwey et al, 1965). The Inaba LPS exhibited full activity in mouse protection tests and the heated antigen was fully capable of inhibiting vibriocidal antibody in the vibriocidal antibody inhibition test. In contrast to the Ogawa LPS, the Inaba antigen protected against both challenges, Inaba and Ogawa equally well.

Kaur and Shrivastava (1965) have also reported on LPS protective antigens from both Inaba and Ogawa serotype vibrios which elicited high titres of vibriocidal antibodies.

Such studies indicate that LPS is at least one of the protective antigens and that the Ogawa serotype appears to have a protective component, not shared with the Inaba.

1.13 The structure of V.cholerae LPS

Jackson and Redmond (1971) performed immunochemical studies on the LPS from strain Inaba 569B. The major components they identified in the LPS were heptose, glucose, glucosamine, mannose and glycerol. Lipid A accounted for 30% of the weight of LPS. The authors were unable to account for 30% of the weight of the LPS. Galactose and 2-keto-3-deoxyoctonate (KDO) which typically links lipid A and the polysaccharide moieties were not detectable and it was suggested that the structure of the "core" LPS of V. cholerae may be fundamentally different from that of the Salmonella species.

Jann et al, (1973) also reported the absence of KDO in V. cholerae LPS and extended their study to confirm that KDO is not present in any of the V. cholerae strains they tested namely, S-forms of Inaba and Ogawa, El Tor and Hikojima, R-forms of Inaba and Ogawa. However, they detected fructose in all these LPS molecules and suggested that KDO may be replaced by fructose. They were also successful in isolating 2-amino-2-6-dideoxyglucose (quinovosamine). The role of this amino sugar in the serological specificity was not defined.

Redmond (1975) detected an oligosaccharide in addition to monosaccharides in the hydrolysed product of Inaba 569B LPS. This oligosaccharide required harsher conditions of hydrolysis to yield monosaccharides and was identified as 4-amino-4,6-dideoxy-D-mannose (perosamine) by ^{13}C NMR studies. The perosaminyl oligomer was thought to represent the main sugar

component of V. cholerae LPS. On further anlaysis of vibrio LPS the presence of another highly unstable sugar, 4-amino-4-deoxy-L-arabinose was detected (Redmond, 1978). The sugar was present only in Ogawa LPS and not in Inaba LPS.

Raziuddin and Kawasaki (1976) analysed LPS extracted from cell walls of Inaba 569B and El Tor Inaba. They found that among the characteristic components of gram-negative bacterial LPS, heptose phosphate was present but galactose and KDO were not detected. On the other hand, fructose was present.

1.13.1 The lipid A component of V.cholerae LPS

In enterobacterial LPS, glucosamine represents the backbone of lipid A, carrying the long chain hydroxy acids in ester or amide linkages (Hammond et al, 1984). The nature and distribution of fatty acids in the LPS of V. cholerae Inaba 569B and El Tor Inaba were of an identical pattern (Raziuddin and Kawasaki, 1976). The most abundant fatty acid detected in the lipid A moiety was 3-hydroxy lauric acid. Further characterisation of the lipid A by Raziuddin (1977) indicated that approximately equal amounts of fatty acids $C_{16:0}$, $C_{18:1}$ and 3-hydroxy lauric acid were involved in ester linkages, but 3-hydroxy myristic acid was the only amide-linked fatty acid.

1.13.1.1 The toxic and immunological properties of V. cholerae lipid A

The LPS and lipid A moieties prepared from V. cholerae El Tor exhibited almost equal endotoxic and anti-complementary

activities (Raziuddin, 1978). It was demonstrated that the lipid A representated the toxic centre of V. cholerae LPS as had been observed with other gram-negative bacterial LPS molecules (Peavy et al, 1973; Rietschel et al, 1975; Mansheim et al, 1978). The toxicity of lipid A from V. cholerae El Tor decreased following alkaline digestion which primarily hydrolyses the ester linked fatty acids of the lipid A portion of LPS. These results indicated that ester-linked fatty acids play a crucial role in toxicity. Luderitz et al, (1973) suggested that the presence or absence of acylated hydroxy fatty acid esters may determine the endotoxicity of the LPS.

1.13.2 The polysaccharide portion of V. cholerae LPS

On mild hydrolysis of V. cholerae LPS Raziuddin and Kawasaki (1976) obtained a water soluble fraction of polysaccharides and a chloroform soluble fraction of lipid A. Raziuddin (1980) characterised the water soluble polysaccharides by fractionating them on a column of Sephadex G-50. The eluates were monitored for phosphorus and carbohydrate. Two main peaks differing in molecular size were obtained from all different strains of V. cholerae LPS tested. When each of these peaks were rerun separately on the same column, both were observed to move in their original positions. Detailed analysis of the two peaks showed that high molecular weight fractions did not contain phosphorus and heptose whereas phosphorus and heptose were detected in the low molecular weight fractions. On the basis of haemagglutination assays the high molecular weight

heptose-free fractions were identified as O-specific side chain and low molecular weight heptose-containing fractions as core polysaccharide regions of the LPS. Glucose, heptose, fructose, phosphate and ethanolamine-phosphate were found to be concentrated in core polysaccharide, whereas mannose, rhamnose, glucosamine, D-quinovosamine and D-perosamine were concentrated in O-specific side chains.

Hisatsune and Kondo (1980) compared the chemical and serological properties of LPS isolated from the smooth (S) and rough (R) forms of cholera vibrios. They observed that the S to R mutation of cholera vibrios involved the total elimination of two component amino sugars of S-form LPS, i.e. quinovosamine and perosamine. This elimination resulted in the loss of O-specificity of S-form LPS and concomitant appearance of serological cross-reactivity, in the passive-haemagglutination inhibition test, among R-form LPS molecules regardless of the serotypes, Inaba or Ogawa, of their S parents.

Kabir (1982) analysed V. cholerae 395 Ogawa LPS after splitting it into polysaccharide and lipid A moieties and detected glucose, heptose, fructose, glucosamine and quinovosamine in the polysaccharide portion. The presence of 4-amino-arabinose in only Ogawa LPS was confirmed by his findings. From gas-liquid chromatography and mass spectrometry on methylated polysaccharide the author was able to detect a branched structure with glucose and heptose residues primarily appearing at the nonreducing-end groups.

Sen et al, (1979), using methylation studies, obtained

results which indicated that the nonreducing ends of LPS extracted from Inaba 569B were occupied by glucopyranosyl and heptopyranosyl residues. They also observed the presence of two types of heptose residues, of which a large amount behaving like D-glycero-L-manno-heptose is located at the nonreducing end of the molecule. One or both heptose residues were thought to be located in the interior of the molecule. One of them had (1→2) linkages and the other was branched. The 2-amino-2-deoxyglucose residue detected was also suggested to be present in the interior part of LPS as (1→4) linked residues.

Sen et al, (1980) identified the two heptoses described above with D-glycero-L-manno-heptose being the major one and D-glycero-L-gluco-heptose the other. The heptoses were assigned β anomeric configurations from oxidation studies, though the presence of the α-anomeric configuration of sugar residues was also indicated by the low specific rotation of LPS.

Majumdar et al, (1982) obtained a single precipitin line in the Ouchterlony double diffusion test with LPS from Ogawa G-2102 and rabbit antisera raised against whole organisms, indicating the LPS to be homogenous. The polysaccharide moiety obtained on acid hydrolysis of LPS was resolved into antigenic and core polysaccharides. Only the antigenic polysaccharide gave a single precipitin line in the ouchterlony gel-diffusion tests. These results corroborated the observations of Raziuddin (1978) and established the presence of both a core region and an O-side chain which was responsible for the serologic specificity of V. cholerae LPS.

The oligosaccharide obtained by Redmond (1978) from hydrolysis of Inaba 569B LPS was analysed by ^{13}C NMR and chemical methods (Redmond, 1979). It was found out to be a regular repeating structure of ∝-(1→2) linked chain of D-perosamine units, with amino groups acylated with 3-hydroxypropionyl groups. Gel chromatography of the polymer suggested a molecular weight of 10,000 which according to the author was equivalent to an unbranched chain of about 60 perosamine residues. Trace amounts of other sugars in the polysaccharide were detected by ^{1}H NMR and these were were presumed to be present at the reducing terminal of the polymer.

Kenne et al, (1982) hydrolysed V. cholerae LPS into lipid A and polysaccharide portions. The polysaccharide was further separated into two fractions of M_r 9000 (major) and M_r 900 (minor) by gel filtration. The main component of the polysaccharide was identified as the unusually acid resistant oligosaccharide and its structure was elucidated by ^{13}C and ^{1}H NMR spectra as a homopolysaccharide composed of (1→2)-linked 4-amino-4,6-dideoxy-∝-D-mannopyranosyl (perosaminyl) residues, the amino groups of which are acylated by 3-deoxy-L-glycero-tetronic acid. The assignment of propionyl residues to the acyl groups by Redmond (1979) was not thought to be correct.

Kenne et al, (1982) found that 60% of saponified LPS and 70% of the polysaccharide consisted of the homopolysaccharide and hence they suggested that the main part of the O-antigen of V. cholerae LPS is composed of simple repeating-units

(perosaminyl) as observed for other gram-negative bacteria. According to the authors the N-acetylated perosaminyl polymer most probably represents the LPS antigenic determinant, A, common to Inaba and Ogawa and this was consistent with their results on haemagglutination-inhibition. No O-antigen was present in the M_r 900 fraction. In conclusion the authors noted that the part connecting lipid A with the O-antigen differed considerably from the cores observed in other gram-negative bacterial LPS molecules.

Brade (1985) reinvestigated the presence of KDO in both Ogawa and Inaba serotypes of V. cholerae because LPS from each serotype was found to be an active inhibitor of the serologic test system specific for the common LPS which consisted of KDO and at least one neutral sugar. KDO in V. cholerae LPS was identified by gas-liquid chromatography and mass spectrometry. From this recent study it was shown that KDO phosphate is a constituent of the LPS of V. cholerae Ogawa and Inaba. This compound is liberated under harsh hydrolytic conditions and can be dephosphorylated subsequently by the action of alkaline phosphatase. The results indicated that at least two different KDO phosphates are present in the hydrolysate. One of them was identified as KDO-5-phosphate, the other as 7- or 8- phosphate. Whether the latter compound represents a constituent of LPS remains to be established. Thus the lack of KDO was shown not to be a taxonomical criterion for the members of Vibrionaceae.

It is clear from the data reviewed above that the structure of V. cholerae LPS is not yet fully understood and many features

remain unresolved.

1.14 Antigenicity of core LPS

Anti-core antibodies have gained importance because of their potential to react with a broad spectrum of gram-negative bacteria, thus offering wide ranging protection for immunosuppressed patients (Marks et al, 1982; Teng et al, 1985; McCabe et al, 1977). Several research groups have now produced MCAbs against some components of core LPS (Nelles and Niswander, 1984; Mutharia et al, 1984; Lind et al, 1985). A new type of common antigen located in the inner core region of LPS has been detected in a variety of gram-negative bacteria (Brade and Galanos, 1983a). Immunochemical investigations have shown the new antigen to be an ∝-2→4 interlinked 3-deoxy-D-manno-octulosonic acid (dOclA) disaccharide (Brade and Galanos, 1983b; Brade and Rietschel, 1984), which has also been detected in the Ogawa and Inaba serotypes (Section 1.13.2). Previous studies have indicated that production of antibodies against lipid A required immunisation of animals with bacteria coated with acid hydrolysed lipid A (Bruins et al, 1977; Galanos et al, 1971; Johns et al, 1977). However, anti-lipid A MCAbs have been raised by Mutharia et al, (1984) by using whole heat-killed E. coli J5 cells for priming mice before removal of their spleens for fusion.

Gustafsson et al (1982) have produced a MCAb directed against the core region of V. cholerae LPS. These antibodies were inhibited by LPS preparations of both serotypes of 01 group of

vibrios and some non-01 group of vibrios as detected on ELISA. Unrelated bacterial LPS molecules were unable to inhibit the MCAb. This according to the authors indicated that the MCAb was directed against an antigen present only in V. cholerae.

1.15 Bacteriocidal activity and LPS

Resistance to complement killing is an attribute of many gram-negative bacteria. The presence of the smooth phenotype, that is, LPS molecules containing long polysaccharide side chains (O-antigen), is the characteristic of the gram-negative cell envelope correlated with serum resistance. This conclusion has been based upon studies comparing rough isogenic mutants with smooth parental strains of E. coli and Salmonella species (Muschel and Larsen, 1970; Nelson and Roantree, 1967; Rowley, 1968). Cells lacking the O-antigen are generally killed directly by complement in the absence of specific antibodies but become resistant to serum when O-antigen is synthesised (Dlabac, 1969). Studies conducted by Sansano et al, (1985) with E. coli suggest that the increased resistance to the lethal action of normal human serum shown by the smooth phenocopy may be due to the blocking of antibody binding sites by the O-antigen of LPS, thereby preventing activation of the classical pathway of complement.

1.16 Polyclonal effects of bacterial LPS on B-cells

It has been suggested that LPS treatment of B-cells might replace or mimick the enhancer in activating certain regions of immunoglobulin gene transcription (Alt et al, 1982). LPS has also been thought to induce the synthesis of an enhancer binding protein required for the activation of kappa transcription (Nelson et al, 1984). A variety of studies have pointed to post-transcriptional effects of LPS treatment on immunoglobulin gene expression (Yuan and Tucker, 1982; Mains and Sibbley, 1982; Yuan, 1984).

LPS molecules containing linear homopolysaccharides consisting of mannose as the O-specific polysaccharide chains have been reported to have exceptionally strong adjuvant activity (Kido et al, 1985; Kato et al, 1985). The O-specific chain of V. cholerae, B. abortus and Y. enterocolitica are also homopolysaccharides but of mannopyranosyl derivatives (Section 1.18). The adjuvant properties of such homopolymers have not been studied in isolation but are of interest in the context of this work.

1.17 Protein antigens of V. cholerae

Neoh and Rowley (1970), from their studies of antisera absorbed by LPS and outer membrane preparation, provided evidence that polysaccharide determinants may not be entirely responsible for the observed vibriocidal activity of antisera produced against V. cholerae. Kabir (1980) used several strains of both

serotypes, Ogawa and Inaba and Kelly and Parker (1981) studied a wild-type strain, CA401 and each isolated and characterised outer membrane preparations. Although they worked with different strains, each group consistently observed a major protein of M_r 45,000 to M_r 48,000. Manning et al, (1982) have analysed the extent of variability of the major proteins in V. cholerae and showed considerable variation in the proteins by using a variety of detergents to solubilise the them, with the exception of a protein of M_r 25K. Manning and Haynes (1984) identified a M_r 25K protein in the outer membrane of vibrio strains which was not present in a number of other organisms. The protein was shown to be very immunogenic. Stevenson et al, (1985) have purified the M_r 25K outer membrane protein of V. cholerae and cloned its structural gene with a view to generating a protein vaccine.

1.18 Cross-reactions of V. cholerae with bacteria of other genera

Many investigators have observed serologic cross-reactions between Brucella and V. cholerae (Mathur, 1960; Eisele et al, 1948). Eisele et al, (1946) and McCullough et al, (1948) attributed the cross-reaction to a flagellar H antigen of V. cholera that is related to a somatic antigen in the three species of Brucella. Gallut (1953) ascribed the cross-reaction to heat-stable somatic O rather than H antigens of V. cholerae. Feeley (1969) examined the antigenic relationship between Brucella species and V. cholerae by agglutinin and agglutinin-absorption tests by using rabbit antisera. He observed

that Brucella antisera agglutinated only the Inaba serotype of V. cholerae at low titres. The Inaba reactive antibody was absorbed by either heat-stable Ogawa or Inaba O-antigens. Cholera antisera from rabbits immunised with either O or HO antigens of either Ogawa or Inaba contained Brucella agglutinins. This activity was absorbed completely from Ogawa antiserum by either Ogawa or Inaba O antigens but only partially from Inaba antisera by Ogawa O antigen. Thus the author concluded that the cross-reactive antigen is a heat-stable O antigen and is more dominant in the Inaba than in the Ogawa serotype of V. cholerae.

Barua and Watanabe (1972) described the production of vibriocidal antibody against V. cholerae, particularly the Inaba serotype, in Y. enterocolitica immunised rabbits or infected men. Y. enterocolitica serotype 0:9 has been regarded as strongly cross-reacting with Brucella species (Ahvonen and Sievers, 1969).

The serological cross-reactivity between Y. enterocolitica serotype 0:9 and the LPS of V. cholerae and Brucella species have now been related to the presence of N-acylated 4-amino-4,6-dideoxy-α-D-mannopyranosyl residues in their respective O-antigenic chains (Caroff et al, 1984a; Caroff et al, 1984b). Bundle et al, (1984) confirmed the serological cross-reactions between B. abortus and Y. enterocolitica with MCAbs. Since V. cholerae 01 shares a very similar O-chain structure with B. abortus and Y. enterocolitica 0:9 differing only in N-acyl grouping the anti-Y. enterocolitica and anti-B. abortus MCAbs were tested with Ogawa and Inaba LPS on

ELISA by the authors. Ascitic fluid derived from the MCAbs failed to reveal significant binding at dilutions of 1 in 100, whereas the binding to B. abortus and Y. enterocolitica was to the order of 1 in 10,000. This contrasted with previous observations with polyclonal antisera (Barua and Watanabe, 1972; Sandulche and Marx, 1978). The authors therefore concluded that although some vestige of recognition existed for the polysaccharide, the nature of the N-acyl group on the backbone sugar was crucial to the binding of these MCAbs.

1.19 Introduction to hybridoma technology

The theory of monoclonal antibody (MCAb) production is based on the clonal selection hypothesis of Macfarlane Burnet (Burnet, 1959). Each mammalian B lymphocyte has the potential to make a monospecific antibody. The constant region of the antibody chain may alter during the differentiation of the lymphocyte clone but the variable region retains the singular specificity.

The first report of hybridoma production was in 1970 (Sinkovirs et al, 1970) with virus specific lymphocytes together with tumour cells and subsequently interspecies (Schwaber and Cohen, 1973) and human (Bloom and Nakamura, 1974) hybridoma products were reported before MCAb technology was established by Kohler and Milstein in 1975. Since then MCAb production has grown exponentially to be utilised in a number of different ways.

The experimental problem encountered in monospecific antibody production relates to the fact that plasma cells which secrete antibody are terminally differentiated lymphocytes with a finite life span and cannot be normally grown in culture. However, tumours of such cells can be found in most animals and, in particular, can be readily induced in mice with the aid of mineral oils. The tumour cells secrete an antibody of single and unknown specificity but can be grown indefinitely in culture. If such tumour cells can be fused with a lymphocyte which makes antibody of the required specifity, the progeny may have the eternal growth capacity of one parent together with the specifity of the other (Kohler and Milstein, 1975, 1976). The technique, however, involves the selection of a mutant strain of the tumour parent line which does not itself secrete antibody so that the production capacity of the progeny is directed to the specific antibody. A second and most important criterion is to select a tumour cell line as a fusion partner which is in some way vulnerable to the cell culture conditions so that it cannot survive unless it has participated in a fusion. The commonest way of achieving this is to use a parent tumour line which lacks either the enzyme thymidine kinase or hypoxanthine phosphoribosyl transferase (HPRT). These are enzymes of the salvage pathway of nucleic acid metabolism and are essential to cells growing in the presence of aminopterin which blocks the main pathways of nucleotide synthesis. After the fusion the cells are therefore usually grown on medium containing Hypoxanthine, Aminopterine and Thymidine (HAT) (Littlefield, 1964) in which any parent

tumour cells which have not participated in a fusion will die.

1.20 Comparison of MCAbs and conventional antiserum

The antigenic determinant is a particular site on an antigen responible for binding the antibody. This involves a few residues of amino acids and or sugars and in particular the specific conformation of the binding region. (Amit et al, 1985). Coventional polyclonal sera raised against an antigen have not only antibodies to several determinants on the antigen but also a family of antibodies of different structure and avidity which compete for each individual determinant. Consequently, extensive cross-reaction may occur between the antibodies and two antigens which have similar determinants (Campbell, 1984). This makes it difficult to analyse two different antigens sharing common determinants with polyclonal serum. On the other hand, identification of similar and dissimilar determinants on two antigens can be readily achieved by the use of MCAbs which can be selected for their ability to bind to a unique determinant or one shared by the required antigens. The fine specifity of a MCAb can be used in a variety of ways, like detecting small differences between two strains of bacteria and utilising these differences in specificities for diagnostic or preparative purposes.

Talmage (1959) pointed out that if it were possible for an antibody to bind two or more different antigens, then the great specificity of polyclonal sera could in effect be statistical. Thus one antibody could have the ability to react with eptopes A,

B and C, another with A,D and E and another with A,F and G, the specificity of the antiserum would then be observed as against A only since the varying cross reactions of the component antibodies would only give a minor background. However, a monoclonal antibody with cross reactive potential might show significant interactions with possibly unrelated antigens. Thus every cross-reaction observed with a MCAb must be analysed with care.

To achieve monospecifity of conventional antiserum, absorption by cross-reacting antigen is necessary. This procedure reduces the titre of the antiserum by removing all antibodies directed towards the target antigen but which also happen to cross-react with the absorbing antigen, as is the case with serologically related bacteria. Again, a hybridoma cell line can be selected for producing high affinity antibodies which exhibit significantly higher titres than polyclonal antiserum. Absorption procedures of antisera may vary from laboratory to laboratory, and are further complicated by the variability of each bleed from immunised animals. These factors make polyclonal antiserum unsuitable for the detection of specific cell surface molecules. Monoclonal antibodies do not have any of these drawbacks since they can be cultured when and where required and each time they will produce well characterised antibodies.

1.21 Monoclonal antibodies to bacteria

MCAbs have been used for studying bacterial antigens by various groups (Nelles *et al*, 1984; Mutharia *et al*, 1984, Bundle

et al, 1984; Gustafsson et al, 1983; Lind et al, 1985 ; Quinn et al, 1984). They have largely been employed as superior reagents for typing different strains of bacteria or for antigen identification and the information obtained has emphasised the considerable potential of MCAbs as general bacteriologicical reagents. Such antibodies have long term potential if it is possible to use them to develop good anti-idiotype vaccines (McNamara et al, 1984).

1.22 The objectives of this study

V. cholera, the etiological agent of cholera harbours a complex cell-surface LPS which determines its serological identity. Chemical analyses of the LPS have shed some light on the possible structures of the LPS antigens of these vibrios (Section 1.12), but the type-specific antigens remain poorly understood and the serological cross reactions among V. cholerae and other gram-negative bacteria (Section 1.18) are difficult to explain.

The presence and absence of cross-reactions between two strains of bacteria may mean much more than just the sharing of chemical groups responsible for serological activity. The accessibility and geometry of the residues on the bacterial surface are also important and these can only be analysed by the use of antibodies, and preferably monoclonal ones.

The study was undertaken in order to try to better define at least some of the major cell surface antigens of V. cholerae by means of hybridoma technology.

MATERIALS AND METHODS

2.1 Materials

The materials used during the course of this study are listed below with the names and addresses of their suppliers.

2.1.1 Cell Culture materials

RPMI-1640 and Aminopterine were supplied by Flow Laboratories, Irvine, Scotland.

Foetal calf serum and Fungizone were obtained from Gibco Ltd., Paisely, U.K.

Penicillin and Streptomycin were obtained from Glaxo Pharmaceutical, London.

Hypothanthine was obtained from Sigma Chemical Co., Dorset, U.K.

Thymidine was obtained from Boehringer-Mannheim, Sussex, U.K.

2.1.2 Disposable Plasticware

Tissue Culture Flasks were supplied by Nunc, Trident House, Paisley, U.K.

Tissue Culture Plates were supplied by Costar Northumbria Biologicals Ltd., Northumbria, U.K.

MicroELISA plates were supplied by Dynatech Laboratories Ltd., Sussex, U.K. Sterile universals were obtained from Sterilin Ltd., Feltham, England, U.K.

2.1.3 Cell lines

Rat myeloma lines Y3.Ag.1.2.3 and YB2/0 were obtained from C.Milstein, MRC, Cambridge, U.K.

2.1.4 Vibrios

V. cholerae serotype Ogawa (NIH 41) and Inaba(35 A3) were obtained from The David Bruce Laboratories, Wiltshire, U.K.

2.1.5 Animals

DA rats were obtained from OLAC (1976) Ltd., Blackthorn, Bicester, U.K. The rats were propagated by sibling mating and entered the study at 3-5 weeks of age.

Lou rats were obtained as a gift from Pathology Veterinary School, Glasgow University.

Lou x DA F1 hybrids were propagated in the animal house of Glasgow University.

2.1.6 Chemicals

Freund's complete adjuvant (H37 Ra) was obtained from DIFCO Laboratories Surrey, U.K. 2,6,10,14-Tetramethyl pentadecane (Pristane) was obtained from Aldrich Chemical Co.Ltd.,

Gillingham, Dorset, U.K. Hypoxanthine, 4-chloronaphthol, Periodic acid, Concanavalin A, Wheat germ agglutinin, Staphylococcus protein A (Cowan strain), Agarose, Polyoxyethylene sorbitan monolaurate(Tween 20), Phenylmethyl sulphonyl fluoride (PMSF), Tris (hydroxymethyl) aminomethane (Trizma Base), Sodium azide and Dimethyl Sulphoxide (DMSO) were obtained from Sigma Chem.Co.Ltd, Dorset, U.K.

Sodium chloride, ammonium chloride, ammonium sulphate, calcium chloride, citric acid and manganous chloride were all of analytical grade and were supplied by BDH Chemicals Ltd., Dorset, U.K.

Polyethylene glycol (PEG) 4000 was obtained from Merck, Hydrogen peroxide was obtained from Fisons, England, U.K.

2.1.7 Electrophoretic Materials

Acrylamide, N,N,N'N'tetramethylene diamine (TEMED), N,N'-methylene bis acrylamide and ammonium persulphate were obtained from B.D.H. Chemicals Ltd., Poole, Dorset, U.K. 2-mercaptoethanol and 2,5-diphenyl oxazole (PPO) were obtained from Koch Light Laboratories, England, U.K. Membrane Filters 0.45um was supplied by Schleicher and Schull, Surrey, U.K.
Low molecular weight marker proteins was supplied by Phamacia Fine Chemicals, Hounslow, Middlesex, U.K.

2.1.8 Stains

Coomassie Blue (R), Coomassie Blue (G) and Naphthol Blue Black were obtained from Sigma Chem.Co.Ltd., Dorset, U.K.
Silver nitrate was obtained from Jhonson Matthey Chemicals, U.K.

2.1.9 Radiochemicals

L-^{35}S- Methionine, specific activity 500Ci/mmole was supplied by Amersham, Bucks., England and ^{125}I-NaI, specific activity 200Ci/mmole was supplied by Western Infirmary, Glasgow University,Scotland.

2.1.10 Photographic Materials

X-ray films (Kodak X-Omat S), SX-80 developer and FX-40 X-ray liquid fixer were supplied by Kodak Ltd., Hemel Hampstead, U.K. 400 ASA Photographic films (Kodak) were purchased from a photographic retailer, Glasgow, Scotland.

2.1.11 Serological Reagents

Rabbit anti-rat IgG (H+L), Rabbit anti-rat IgG (H+L) horse-radish peroxidase, Rabbit anti-rat IgG (H+L) conjugated to fluorescein, Sheep anti-rat IgG1, Goat anti-rat IgG2a, rabbit anti-rat IgG2b, goat anti-rat IgG2c were supplied by Miles Laboratories Ltd., Slough, U.K.
Normal goat serum, normal rabbit serum and donkey anti-rabbit precipitating serum were obtained from Scottish Antibody Production Unit, Wishaw, Scotland, U.K.

2.1.12 Enzymes

Ribonuclease A (from bovine pancreas), α-Mannosidase(jack bean), β-Glucosidase (bitter almonds) Type I, α-Glucosidase (yeast) Type VI, α-Glucosidase (rice) Type V were obtained from Sigma Chemical Co. Ltd., Dorset, U.K.

Proteinase K was obtained from Boehringer-Mannheim, Sussex, U.K.

2.1.13 Miscellaneous

Lipopolysaccharides of *Salmonella typhimurium*, *E. coli* 0111:B4, *E. coli* 0127 B8 and bovine serum albumin was supplied by Sigma Chemical Ltd., Eppendorf tubes (1.5ml capacity) were obtained from Eppendorf, Geratebau, Netheler and Hinz, U.K. Suppliers Anderman and Co., Surrey, U.K. Dialysis tubing was obtained from Visking Tubing, Scientific Instruments Centre Ltd., London. Multichannel micro-pipettes were obtained from Flow Laboratories, Ayrshire, U.K. Micro-pipettes used during the course of this study were obtained from Gilson Anachem Ltd., Luton, U.K.

All other reagents were of analytical grade or of the highest available purity.

2.2 Standard Solutions and Buffers

2.2.1 Fusion and Cell Culture Media

2.2.1.1 RPMI-1640 medium

This medium was supplied as a dry powder by Flow Laboratories. The medium was made up from the supplied powder by the cell culture unit of Glasgow University as instructed by the suppliers. Sterilisation was done by filtration and then aliquoted and stored at 4^0C.

The composition of RPMI-1640 medium is shown in Table 2.1.

2.2.1.2 Heat-Inactivation of foetal calf serum (FCS)

Foetal calf serum was inactivated at 56^0C for 30 minutes and stored at -20^0C. Frequent freeze-thawing was avoided.

2.2.1.3 HAT and HT media

The concentration of HAT and HT in medium was as described by Litlefield (1964). Hundred fold concentrated HT stock was prepared by dissolving 136.1mg hypoxanthine, 38.7mg thymidine and 2.25mg glycine in 100ml of distilled water. Hypoxanthine was solubilised by stirring the mixture at 50^0C for 60 minutes. The solution was then sterilised by filtration and aliquoted and stored in dark at -20^0C.

HT medium was made up by diluting 1.0ml of the sterilised stock solution with 100ml of complete medium (Section 2.2.1.5) to give a concentration of $3x10^{-6}$M, $1x10^{-4}$M and $1.6x10^{-5}$M for glycine, hypoxanthine and thymidine respectively.

HAT medium was prepared with the addition of 0.4ml of stock aminopterin (0.1mM) to 100ml of HT medium to give a final

Table 2.1 Composition of RPMI 1640 medium

Amino acids	mg/litre
L-Arginine	200.00
L-Aspargine H20	56.82
L-aspartic acid	20.00
L-Cystine,disodium salt	59.15
L-Glutamic acid	20.00
L-glutamine	300.00
Glycine	10.00
L-Histidine	15.00
L-Hydroxyproline	20.00
L-Isoleucine	50.00
L-Leucine	50.00
L-LysineHCl	40.00
L-Methionine	15.00
L-phenylalanine	15.00
L-Proline	20.00
L-Serine	30.00
L-Threonine	20.00
L-Tryptophan	5.00
L-Tyrosine	20.00
L-Valine	20.00
Vitamins	
Biotin	0.20
D-Calcium pantothenate	0.25
Choline chloride	3.00
Folic acid	1.00
i-Inositol	35.00
Nicotinamide	1.00
p-aminobenzoic acid	1.00
PyridoxineHCl	1.00
Riboflavin	0.20
Thiamin.HCl	1.00
Vitamin B12	0.005
Inorganic salts	
$Ca(NO_3)_2$	69.49
KCl	400.00
$MgSO_4.7H_2O$	100.00
NaCl	6000.00
$NaHCO_3$	2000.00
$Na2HPO_4$	800.7
Other Compounds	
Glucose	2000.00
Glutathione	1.00
Sodium phenol red	5.00

concentration of $4x10^{-7}$M.

2.2.1.4 Antibiotics

Penicillin 10^5 I.U./L and streptomycin 100mg/L were used in routine culture medium.

2.2.1.5 Complete Medium

This was made up with the addition of either 10% or 20% FCS (v/v) and fungizone (2.5µg/ml) to RPMI-1640 and referred to as 10% or 20% complete medium.

2.2.1.6 Trypan Blue Dye Solution

One part of 1% (w/v) trypan blue dye solution was mixed with four parts of 1% (w/v) NaCl. The mixture was then diluted with saline to give 0.1% solution immediately before use for the Trypan Blue exclusion test.

2.2.1.7 Polyethylene glycol (PEG) solution

40% (w/v) PEG was made up by adding 40g of PEG to 60ml of RPMI. The mixture was solubilised and sterilised by autoclaving at 15lb/sq.in. for 15 minutes. The mixture was aliquoted when hot and stored at -20^0C.

2.2.1.8 Ammonium chloride solution

0.9g of ammonium chloride was dissolved in 100ml of distilled water to give 0.9% solution. This was then aliquoted in volumes of 300µl and stored at -20^0C.

2.2.2 Immunoassay Buffers

McIlvaine's Buffer

Stock solutions

Citric acid (0.1M)

$Na_2HPO_4.12H_2O$ (0.2M)

Working diluent

17.9ml of citric acid and 32.1ml of Na_2HPO_4 were mixed and the volume made up to 100ml to give a solution of pH6.0.

2.2.3 Saline Buffers

2.2.3.1 Phosphate buffered saline (PBS) PH7.4

NaCl (145mM)

Na_2HPO_4 (9mM)

NaH_2PO_4 (1.3mM)

2.2.3.2 PBS-Tween

0.5ml of Tween 20 was added to every litre of PBS.

2.2.3.3 Tris-saline buffer pH7.2

Tris-HCl (20mM)

NaCl (0.15M)

NaN_3 (0.05%)

2.2.3.4 Tris-Tween buffer

5.0ml of Tween 20 was added to every litre of tris-saline buffer.

2.3 Methods

2.3.1 Animal Handling

2.3.1.1 Immunisation of rats

DA rats were immunised with either Ogawa (NIH 41) or Inaba (35 A3) serotypes of Vibrio cholerae. The vibrios were obtained as a suspension in 0.5% phenol-saline. The organisms were washed in PBS and resuspended in PBS. 10^9 vibrios in 0.05ml of PBS were mixed with equal volume of complete Freund's adjuvant and vortexed to give a homogenous emulsion. This emulsion was injected subcutaneously at two sites into a DA rat. A week later the process was repeated. The rat serum was tested for antibodies against the respective vibrios 4-5 days after the second injection.

Any rat that had a high titre i.e. atleast a titre of 1 in 1000, was given an intraperitoneal boost of $5x10^9$ vibrios in PBS 4 days before the fusion.

2.3.1.2 Collection of Blood from Rats

Immunised rats were bled by cutting the end of the tail by a sharp scalpel blade. A few drops of blood were collected by letting it drip from the cut end into an eppendorf tube and it was allowed to clot. The clot was centrifuged and serum obtained.

Rats used for the fusions were sacrificed in an ether jar. The blood was then immediately collected from the inferior vena cava using a G21 needle.

2.3.1.3 Pristane priming of rats

2ml of pristane were injected intraperitoneally into rats, 7-10 days before injecting hybridoma cells into them.

2.3.1.4 Induction of ascites in rats

$2\text{-}5\times10^{6}$ hybridomas were washed several times in PBS to remove any traces of albumin carried over from the tissue culture medium. The cells were finally suspended in PBS and injected intraperitoneally into a rat.

2.3.1.5 Collection of ascites fluid

The rats developed visible ascites tumours 3-5 weeks after injection with hybridomas. The ascitic fluid was collected by sacrificing the rat in an ether jar and withdrawing the fluid with a syringe fitted with a G21 needle.

2.3.2 Production of rat-rat hybrid myelomas

2.3.2.1 Rat Myelomas used for Hybridoma production

The rat cell lines Y3.Ag 1.2.3 (Y3) and YB2/3AG20 (YB2/0) described in Section 1.20.1 were used for the production of the B-cell hybridomas.

2.3.2.2 Culture of the Myeloma Lines

The myeloma cells to be used for a fusion were grown in a spinner culture flask in 10% complete medium at 37^0C in a water bath equipped with a magnetic stirrer. The flasks were filled with 5% CO_2. The cells required for a fusion were collected from the spinner culture medium and examined for atleast 95% viability (described in Section 2.3.2.3).

2.3.2.3 Cell viability and cell count

Cell viability was determined by Trypan Blue exclusion test. An aliquot of cell suspension was mixed with an equal volume of dye solution. The number of live and dead cells were counted in a Neubauer Haemocytometer.

2.3.2.4 Preparation of spleen cell suspension

DA rats were sacrificed in an ether jar. The rats were then soaked in 70% propanol and dissected using sterile equipment. The blood was collected immediately as described in Section 2.3.1.2. The spleen was then removed and placed in a

sterile universal. Further work with the spleen was carried out in a sterile hood.

The spleen was teased apart with two G21 needles in a petridish containing RPMI. The released spleen cells were passed through a G21 needle twice and twice through a G25 needle to give a single cell suspension. The cells were then washed and resuspended in RPMI and counted as described in Section 2.3.2.5.

2.3.2.5 Spleen cell count

50µl of spleen cell suspension was pipetted into an eppendorf tube containing 300µl of 0.9% ammonium chloride and incubated for 5 minutes at room temperature. The lymphocytes were counted in a Neubauer Haemocytometer.

2.3.2.6 Fusion Protocol

Fusion between spleen and myeloma cells was carried out essentially by the procedure of Kohler and Milstein (1975) with minor modifications (Campbell,A.M. 1984).

1ml of 20% complete medium was dispensed into each well of a 24-well costar plate. The number of costar plates used depended on the number of spleen cells obtained from a spleen. Usually 4 plates were set up for a single fusion.

Myeloma cells collected from a spinner culture were washed and resuspended in RPMI. A small aliquot of both spleen and myeloma suspension were kept aside to be used as controls. The remaining spleen and myeloma cells were mixed at a ratio of 2:1 and centrifuged at 500xg for 5 minutes. The supernatant was

discarded and the pellet was dispersed by gentle tapping. 2ml of 40% w/v PEG was added to the pellet over a period of 30 seconds with gentle shaking. The pellet was then resuspended for 30 seconds and incubated at room temperature for another 30 seconds. 5ml of RPMI was then added to the resuspended mixture over a period of 90 seconds and a further 5ml of RPMI all at once. The mixture was then incubated at room temperature for 2-3 minutes and centrifuged at 500xg for 5 minutes.

The cells were then suspended at a concentration of $2x10^7$ spleen cells/ml of complete medium containing 20% foetal calf serum. 50µl (a drop) of resuspended cells were dispensed into each well containing complete medium.

2.3.2.7 Spleen cell feeder layer

A spleen cell suspension was prepared as described in section 2.3.1.4 in complete medium containing 20% foetal calf serum at a concentration of 10^5 cells/ml. 100µl of this suspension was dispensed into each well of a 96-well costar plate.

2.3.2.8 Cloning by limiting dilution

2-4 weeks after the fusion hybrid clones emerged in some of the wells. When the clones were quite confluent the supernatant was tested for antibody secretion on an ELISA. The positive clones were then cloned by limiting dilution. This was carried out by suspending the hybrid cells at 10 cells/ml in complete medium containing 20% foetal calf and dispensing 100µl

into wells prelayered with feeder cells. The suspension of hybrid cells was then double diluted and 100µl was again dispensed into each well. The doubling dilution was carried out once more so that finally 0.25 cells were dispensed into the wells.

Positive clones from the first cloning were cloned for the second and third time by the same procedure. Positive clones from the lowest dilution were expanded in flasks and some were frozen down.

2.3.2.9 Freezing and Thawing of cells

Cells were suspended in 1.0ml aliquots containing $2x10^6$ cells in RPMI supplmented with 20% foetal calf serum and 10% DMSO in 1.5ml capacity cryostat ampoules. The aliquots were frozen at -70^0C overnight, then transferred to a gas-phase liquid nitrogen freezer.

Cells brought up from liquid nitrogen were quickly thawed in a 37^0C water bath, washed twice with RPMI, suspended in complete medium containing 20% foetal calf serum and incubated at 37^0C in a humidified CO_2 incubator.

2.3.2.10 Routine culture of hybridoma cells

All hybrid cells were cultured in complete medium containing 20% foetal calf at 37^0C in a humidified CO_2 incubator.

The clones established after the third cycle of limiting dilution cloning were regarded as monoclonals. The monoclonals were propagated either in spinner cultures or as ascitic fluid (Section 2.3.1.4).

2.3.3 Immuno Assays

2.3.3.1 Enzyme Linked Immunoabsorbent Assay (ELISA)

Solutions

i)Blocking buffer

2% Bovine Serum Albumin

3% Normal goat serum in PBS

ii)Antiserum/Conjugate dilution buffer

0.5% bovine serum albumin in PBS

iii)Substrate

0.4mg/ml o-phenylene diamine dissolved in McIlvaines buffer containing 0.32µl/ml hydrogen peroxide(0.01%)

Routine Screening ELISA

96 well microtitre plates (Dynatech MicroELISA Systems) were first washed with distilled water and then coated with 10^6 vibrios/100µl/well in PBS. The plates were centrifuged at 800xg for 5 minutes at room temperature. The supernant was removed by flicking the plates which were then washed twice with PBS and incubated at room temperature for 30 minutes with blocking buffer to saturate the unreacted sites in the wells. The blocking buffer was removed and the plates were washed three times with PBS-Tween. 100µl of hybridoma supernant or antiserum at the appropriate dilution were then added to each well and the plates were incubated for 2 hours at room temperature and then washed 3 times with PBS-Tween. 100µl of a 1 in 1000 dilution of horse

radish peroxidase conjugated detection antibody (Miles HRP conjugated anti-rat IgG (H+L)) in conjugate dilution buffer were then added to each well and the plates were incubated for a further hour before being washed four times with PBS-Tween. 100µl of substrate were then added and the plate was incubated at room temperature in the dark for 20 minutes. The reaction was stopped by the addition of 50µl of 4N H_2SO_4. The optical density of each well was measured with a titretrek Multiskan spectrophotometer at 492nm.

Assays were performed in duplicate. Controls were wells without vibrios to check for non-specific binding of the antibody to the plate and wells with pre-immune serum or negative tissue culture supernant to check for the non-specific binding of the second antibody to the vibrios. A positive sample was judged to be one 100% in excess of the average control reading.

2.3.3.2 Competition Assay (ELISA)

The competition assay was carried out to confirm the specificity of the antibodies towards respective serotypes of V. cholerae.

The MCAbs were titred on serotypes of vibrios to which they exhibited an apparent specificity. The limiting concentration of antibody able to react with 10^6 vibrios was determined. All six MCAbs were used at their respective limiting concentrations for the competition assays.

ELISA plates were coated with either serotype of V. cholerae and blocked as described in Section 2.3.3.1. Crude

LPS from either serotype or LPS obtained from Sigma or whole vibrios were used as competing antigens. In eppendorf tubes 100µl of varying concentrations of LPS or vibrios were added. To each of these tubes was added 100µl of twice the limiting concentration of a MCAb. The tubes were incubated overnight at 4^0C. 100µl of this mixture was then added to wells of precoated and blocked ELISA plate and the plate was incubated for 2 hours at room temperature. At the end of the incubation period the plates were treated as for routine ELISA assay described in Section 2.3.3.1.

2.3.3.3 Immunofluorescence Assay

Multispot microscope slides were washed with methanol and air dried. 10µl of suspension at 10^8 organisms per ml in PBS were applied to each spot. The slides were allowed to dry at 65^0C for 30 minutes. The vibrios were then fixed in alcohol for 2 minutes. 10µl of MCAb in tissue culture supernatant were then applied to each spot. The control was negative tissue culture supernatant. The slides were incubated in a humidified atmosphere at room temperature for three hours, washed in PBS and reacted with 10µl of fluorescein conjugated rabbit anti-rat IgG (H+L) at a dilution of 1:28. After one hour of incubation in a humidified atmosphere at room temperature, the slides were washed in PBS and examined in a Leitz Orthoplan fluorescent microscope equipped with a Leitz MPV compact photometer.

2.3.3.4 Agglutination Assay

2.3.3.4.1 Slide agglutination assay

10µl of $1.6x10^{11}$ vibrios per ml in PBS were dispensed on a slide and 10µl MCAb concentrated by ammonium sulphate fractionation (Section 2.3.4.3) was added to it and mixed by gentle rocking of the slide.

2.3.3.4.2 Slide agglutination with S.aureus coated antibodies

S. aureus cells were coated with MCAbs as described by Kronvall, 1973. 10^6/ml S. aureus organisms were washed three times with PBS. 1ml of the washed suspension of cells were incubated with 1ml of ten times concentrated MCAb or 1ml of normal rat serum for one hour at room temperature. The incubated cells were washed again with PBS and used for slide agglutination as described in 2.3.3.4.1.

2.3.3.4.3 Agglutination in V-bottom plates

MCAb concentrated ten fold by ammonium sulphate fractionation was used for the assay. 20µl of a suspension of $1.6x10^{11}$ vibrios per ml in PBS were dispensed into each well of a V-shaped 96 well microtitre plate (Costar) and 20µl of MCAb were added to it. The plate was tapped gently to allow thorough mixing and incubated in a humidified atmosphere overnight at room temperature.

Secondary agglutination was accomplished by adding 10µl of rabbit anti-rat IgG (H+L) at a dilution of 1:10 to the

above mixture.

2.3.3.4.4 Agglutinations at altered pH

Buffers

Citrate Buffer pH6.0

9.5 ml of 0.1M citric acid

41.5 ml of 0.1M sodium citrate, total volume was made upto 100ml with distilled water.

Carbonate-Bicarbonate Buffer pH9.2

4ml of 0.2M anhydrous carbonate

46ml of 0.2M of sodium bicarbonate, total volume was made upto 100ml with distilled water.

1.6×10^{11} vibrios/ml were washed five times with either with citrate buffer or carbonate-bicarbonate buffer. Ten times concentrated MCAbs were dialysed overnight at $4^{0}C$ with either of the two buffers. The dialysed MCAbs and the equilibrated vibrios were used for agglutination tests as described in 2.3.3.4.3.

2.3.3.5 Complement fixation assay

Stock Solution

81.6g NaCl (1.4M)

12.1g Tris (0.1M)

33ml of 0.15M of $MgSO_4$

15ml of 0.1M $CaCl_2$

The pH was adjusted with HCl to 7.4 and the final volume was made upto 1 litre.

Working Solution

1g of BSA was dissolved in 100ml stock solution and the final volume was made upto a litre.

Procedure

Tubes were set up with varying concentration of antibody and 10^9 vibrios of either serotype. Controls consisted of antibody at all concentrations with no antigen and antigen with no antibody. 0.6ml of guinea pig complement (1 in 600) were then added to each tube and the tubes were incubated at 4^0C overnight.

The following day the sheep red blood cells (SRBC) (0.1 packed volume) were washed with working solution and resuspended in 10ml of the same solution. 10µl of haemolysin were added to it, mixed well and incubated for 30 minutes at 37^0C. 0.2ml of this SRBC preparation was added to each tube and incubated for about 15-20 minutes watching 100% lysis control. When controls were all lysed, the tubes were cooled quickly and centrifuged 1000xg for 10 minutes. The optical density of the supernatants were measured at 413nm using 0% lysis as blank.

2.3.3.6 Direct binding assay

Blocking Agent

Neat normal goat serum

Linbro multi-well plates were washed with PBS before adding 10^9 vibrios to each well. The plates were then centrifuged at 500xg for 15 minutes. The contents of the plates were discarded and the plates were washed twice more with PBS before adding blocking agent to it. After 30 minutes of incubation the

plate was washed twice with PBS-Tween and 50µl of labelled antibodies along with 50µl of normal goat serum were added to the respective wells. The contents of the plate were discarded after two hours of incubation at room temperature, plates were washed 5-6 times with PBS-Tween and the wells were cut out for being counted on LKB 1275 Minigamma counter.

No antigen in a well served as a control.

2.3.3.7 Inhibition radioimmunoassay

Blocking Agent

Neat normal goat serum

Linbro multi-well disposable trays were used for this assay. 100µl of 10^9 vibrios per well in PBS were dispensed into each well of the plate and the plates were centrifuged at 500xg for 15 minutes. The contents of the wells were discarded with a flick and the wells were washed twice with PBS. Normal goat serum as blocking agent was added to fill up each well upto the brim and incubated for 30 minutes. The plates were washed twice with PBS-Tween. 100ul of cold antibody was added and incubated for two hours, plate was washed again and 50µl of normal goat serum was added before the addition of 50µl of hot antibody. The plate was incubated for another 2 hours at room-temperature. After the incubation the plates were washed 5-6 times with PBS-Tween. The wells were then cut out and counted in the LKB 1275 Minigamma counter.

2.3.4 Immunological and Immunochemical Methods

2.3.4.1 Internal Labelling of Monoclonal Antibodies

Buffers and Solutions

10xTKM Buffer

1M Tris-HCl pH8.2

1M KCl

50mM $MgCl_2$

3D-TKM Buffer

1ml Tritonx100, 1g sodium deoxycholate, 0.5g SDS and 10ml 10xTKM buffer made upto 100ml with distilled water.

Primary Lysis Buffer

10ml of Tritonx100 and 10ml of 10xTKM made upto 100ml with distilled water.

Secondary Lysis Buffer

0.2g deoxycholate, 0.1g of SDS and 2ml of 10xTKM made upto 20ml with distilled water.

Triton stock solution

10% Triton in TKM

1M Sucrose solution in 3D-TKM buffer

0.5M Sucrose solution in 3D-TKM buffer

Immunoprecipitate Buffer

50mM Tris pH8.8, 1% w/v SDS and 0.01%w/v phenol red.

2.3.4.1.1 Procedure

Cells to be labelled were grown at a logarithmic rate. 2-3x10^6 cells were pelleted and washed twice in serum free medium and twice in labelling medium. The cells were then resuspended in 0.25-0.5ml of labelling medium and 50µCi of ^{35}S-methionine were added to it. The cells were incubated at 37^{0}C for 90 minutes. The cells were then centrifuged at 500xg and resuspended in 0.5ml of complete medium. The resuspended cells were divided into equal aliquots. One of the aliquots were incubated at 37^{0}C for 4 hours to study the secretory immunoglobulins of the hybridomas. The other aliquot of cells were lysed to study the nascent intracellular immunoglobulins synthesised by the hybridomas.

2.3.4.1.2 Lysis of Cells

The aliquot of radio-labelled cells to be lysed were washed twice in ice-cold TKM buffer and resuspended in 10µl TKM buffer. The cells were solubilised with the addition of 0.45µl of primary lysis buffer. The mixture was left to stand on ice for 20 minutes. The cells were centrifuged at 3000xg for 20 minutes to pellet the nuclei. The post-nuclear supernatant was transferred to fresh tubes containing 50µl of secondary lysis buffer. Insoluble debri was removed by high speed centrifugation (30,000xg for 30 minutes). The supernatant was collected and the intracellular immunoglobulins precipitated as described below.

2.3.4.1.3 Immunoprecipitation of Monoclonal Antibodies

50ul of the intracellular extract was added to 10µl of goat anti-rat anti-serum (1:10). The mixture was incubated for 1 hour at room-temperature and 50 µl of donkey anti-goat precipitating anti-serum was added to it and incubated overnight at room temperature.

2.3.4.1.4 Isolation of secreted Monoclonal Antibodies

The cells incubated for 4 hours were centrifuged in Sorvall at 15K rpm to pellet the cells. The supernatant was used to immunoprecipitate the monoclonal antibodies as described in Section 2.3.4.1.3.

2.3.4.1.5 Purification of Immunoprecipitates

The immunoprecipitates formed were pelleted at 3,000xg for 30 minutes and resuspended in 100µl of 3D-TKM buffer. Sucrose gradients were prepared by layering 0.5ml of 1M sucrose at the bottom of an eppendorf and 0.5ml of 0.5M sucrose on top of the 1M sucrose layer. 100µl of the resuspended immunoprecipitates were carefully layered on top of the sucrose gradient and centrifuged at 3000xg for 30 minutes and the gradients rinsed with immuno-precipitate buffer. The precipitates were drained with a drawnout pasteur pipette and dissolved in 100µl of TKM buffer. The samples were then applied on 10% SDS-polyacrylamide gels.

2.3.4.1.6 Analysis of the immunoprecipitated Monoclonal Antibodies on SDS-PAGE

30µl of the immunoprecipitated monoclonal antibodies dissolved in TKM buffer were taken up in 30µl of SDS-PAGE sample buffer (see Section 2.3.5.4) and applied on a 10% SDS-polyacrylamide gel. The gel was run at 30mapms till the dye had reached the end of the gel.

2.3.4.1.7 Fluorography

The gel obtained from Section 2.3.4.1.6 was incubated in DMSO for 20 minutes. The process was repeated with fresh DMSO. The gel was then placed in a solution of 60g of PPO dissolved in 250 ml of DMSO and incubated for another 40 minutes. The gel was finally washed in water for 45 minutes and dried. The dried gel was exposed and developed as described in Section 2.3.4.5.2.

2.3.4.2 Ouchterlony

Solutions

1% w/v agar in PBS

Glass slides were coated with 3.5ml of hot agar solution (45^{0}C) and the agar was allowed to solidify. Templates were used to punch holes in the agar. The plugs of agar from the wells were removed using a Pasteur pipette attached to a vacuum line. 10ul of antiserum, control serum and monoclonal antibodies at dilutions of neat, 1/2, 1/4, 1/8, 1/16 and 1/32 were pipetted into the surrounding wells in order of concentration. Monoclonal

antibodies used as neat were 10 fold concentrated from culture supernatant by ammonium sulphate precipitation(Section 2.3.4.3). Rat immunoglobulin class specific antisera was pipetted as neat (10µl/well) in the centre well. The slides were incubated in a humidified atmosphere overnight at room temperature and the precipitin lines were allowed to develop for 3-4 days in a humidified atmosphere at 4^{0}C.

2.3.4.2.1 Staining precipitin lines

Stain

0.025% w/v Coomassie brilliant blue R in methanol:water:acetic acid 50:45:5 by volume.

Destain

Water:acetic acid:methanol 87:8:5 by volume.

Wash solution

5% sodium citrate

Procedure

The slides were washed in 5 changes of wash solution over 48 hours to solubilise any non-specific precipitates and carefully dried with a sheet of filter paper. Slides were stained by immersing them in the stain for 15-20 minutes. They were destained in the destain until the background was clear. The slides were dried by blotting with a filter paper.

2.3.4.3 Fractionation of Antibodies

Monoclonal antibodies were precipitated from the culture supernatant by using 40% saturated ammonium sulphate.

Culture supernatant was cooled to 4^0C in ice and half its volume of saturated ammonium sulphate was added dropwise with constant stirring at 4^0C. The mixture was left on ice 30 minutes and then centrifuged at 500xg for 10 minutes. The pellet obtained was washed twice in 40% saturated ammonium sulphate solution before dissolving it in a small volume of PBS. The solution was dialysed against 100 volumes of PBS at 4^0C overnight to remove traces of ammonium sulphate. The buffer was changed the next day and the dialysis continued for another 2 hours. The sample was collected and stored at -20^0C.

2.3.4.4.Purification of Monoclonal Antibodies from cell culture supernatant

2.3.4.4.1 Purification on DEAE Affi-gel Blue column

Buffer A

0.02M K_2HPO_4, pH 8.0,0.02% NaN_3

Monoclonal antibodies in cell culture supernatant were isolated from albumin in the medium by using a DEAE Affi-Gel Blue column.

A column of DEAE Affi-Gel Blue was prepared of 20ml bed volume. The column was washed with two bed volumes of buffer A. The monoclonal antibody in the culture supernatant was dialysed for 24 hours in buffer A at 4^0C. 2ml of the dialysed neat culture supernatant was applied to the column. The IgG fraction was eluted with two volumes of Buffer A. Fractions of volume 1ml were collected and the protein peak was determined by absorbance

280 nm. Each fraction was tested by ELISA and the activity peak determined. Fractions with the highest activity and the lowest protein content were pooled and lyophilised.

The proteins bound to the column were eluted with two bed volumes of 1.4M NaCl in buffer A. The activity and protein profile of these fractions were also recorded. The column was regenerated with two bed volumes of 6M guanidine hydrochloride, followed by two bed volumes of buffer A.

2.3.4.4.2 Purification of immunoglobulins on QAE-Sephadex

Buffer

0.1M Tris-HCl pH 6.5

QAE-Sephadex A50 was swollen for 3 days in 0.1M Tris-HCl buffer and the suspension was degassed before pouring it into the column. A 10ml column was set up and equilibrated with buffer at a flow rate of 1ml/min. 1ml of ten times concentrated immunoglobulin (Section 2.3.4.3) was layered on top of the column and 4-5ml fractions were collected from the column at about 1ml/min. The protein content of the fractions were measured by their absorbance at 280nm and fractions were collected till the absorbance reached a value of 0.01. Each fraction was tested for their antibody activity on ELISA. Fractions with the highest acitvity were pooled and lyophilised. The sample was regenerated with distilled water and dialysed against 0.1M Tri-HCl before further use.

2.3.4.5 Immunoblotting

LPS or proteins transferred onto the nitrocellulose paper were detected by the anti-sera or monoclonal antibody either by enzyme system or protein A.

2.3.4.5.1 Immunoblotting (Enzyme detection method)

The antigens transferred on the nitrocellulose paper were detected using enzyme-conjugated antisera as described by Towbin et al, (1979).

Solutions

Blocking buffer

5% normal goat serum in PBS

Substrate

Stock solution

0.3g of 4-chloro-1-naphthol dissolved in 100ml of methanol.

Working solution

1 volume of stock chloronaphthol added to 5 vlumes of PBS and final concentration of hydrogen peroxide was 0.01%.

Procedure

After electroblotting the nitrocellulose paper was incubated in the blocking buffer overnight at 4^0C or at 37^0C for an hour to block the remaining reactive sites on the paper. The paper was then incubated with either anti-sera diluted in the blocking buffer or monoclonal antibody in cell culture medium for 1.5-2 hours. The paper was washed after the incubation period with 5 changes of PBS for 30 minutes. Rabbit anti-rat IgG (H+L) horse-radish peroxidase diluted in the blocking buffer (1 in

1000) was applied to the nitrocellulose paper and incubated for another one hour. After the incubation the paper was again washed in PBS five times for 30 minutes. The substrate was then added to the paper and incubated for 2-5 minutes. The bands detected by the antibodies were stained purple. The stained paper was preserved by washing it thoroughly in distilled water and drying it between filter papers. The dried paper was photographed within a week.

2.3.4.5.2 Immunoblotting with Protein A

The procedure followed was that described by Batteiger et al (1982) with minor modifications developed in our laboratory.

Blocking Buffer

0.5% Tween-20 in Tris buffer

Procedure

After the electrophoretic transfer from a gel to nitrocellulose paper, the paper was incubated in the blocking buffer as described in Section 2.3.4.5.2. The anti-sera diluted in the blocking buffer or monoclonal antibody was applied to the paper and incubated for 1.5-2 hours. After the incubation period the paper was washed in blocking buffer five times for 30 minutes. The paper was then incubated with rabbit anti-rat IgG (H+L) diluted 1 in 2000 in the blocking buffer for 1 hour. The paper was washed again in blocking buffer for 30 minutes with 5 changes. Iodinated protein A (10^7cpm) was applied to the paper and incubated for another hour. The unreacted protein A was then

discarded and the paper was washed as described above and dried between filter papers. The dried paper was then exposed on an X-ray film. This was done by placing a film on an intensifying screen in a cassette and then placing the paper over the film. The assembly in the cassette was wrapped in black bags and the film exposed at -70^{0}C till it was ready to be developed. The film was developed and fixed in the dark according to the instructions of the suppliers for the developer.

2.3.4.6 Iodination of Protein A, Immunoglobulins and Lectins

1mg of iodogen was weighed into a vial and dissolved in 0.5ml of chloroform. The chloroform was allowed to vaporise slowly so as to leave a thin coating of iodogen in the vial.

1mg of protein A, 1-0.5mg purified immunoglobulin (Section 2.3.4.4.2) or 0.5mg of lectin was disolved in 0.5 ml of the buffer in which it would be finally used. The solution was transferred to the vial containing the dried iodogen. 400-500µCi of $Na^{125}I$ were added to the vial and incubated at room temperature for 15 minutes with intermittent shaking. After the incubation period the mixture was removed and applied onto a 10ml sephadex-G25M column equilibrated with the appropriate buffer. 1ml fractions were collected and 10ul of each fraction was counted on a LKB 1275 Minigamma counter. The frations with the peak ^{125}I incorporation were pooled and aliquoted if necessary.

2.3.4.7 Radiolabelled-Lectin Binding Studies

Solutions

50% methanol

Fixing Solution

0.2ml of 25% glutaraldehyde in 50% methanol

Tris-KCl Buffer

20mM Tris pH 8.0

0.12MKCl

0.05% NaN_3

Reducing Solution

15mg of $NaBH_4$ in 150 ml of Tris-KCl buffer.

Reaction Buffer

1mM $MnCl_2$

1mM $CaCl_2$

0.1% haemoglobin and 1% Tween 20 in Tris-KCl buffer.

Sonicated organisms or crude LPS from V. cholerae were run on SDS-polyacrylamide gels as described in Section 2.3.5. The gels or the transfers on the nitrocellulose papers were reacted with ^{125}I-Lectins. The whole process was carried out at room temperature.

2.3.4.7.1 Lectin binding on Gels

Gels to be reacted ^{125}I-Lectin was incubated twice with 50% methanol for 30 minutes and fixed with the fixing solution for 90 minutes. The fixing solution was replaced with reducing solution and incubated for 2 hours with two changes of the solution. The gel was then equilibrated with reaction buffer for 1 hour with 4

changes of the buffer. 10^7 cpm of ^{125}I-lectin in the reaction buffer was applied to the gel and shaken for 3 days. The gel was washed for 5 days with 3 changes of reaction buffer each day. The washings were monitored and the gel dried when the counts in the washings were that of the background. The gel was exposed as described in Section 2.3.4.5.2

2.3.4.7.2 Lectin binding on Nitrocellulose Papers

The method followed was identical to that described in Section 2.3,4.7.1 except that the nitrocellulose paper was not incubated in 50% methanol.

2.3.5 SDS-PAGE of sonicated bacteria and LPS

2.3.5.1 Sonication of vibrios

V. cholerae of either serotype were washed in water to remove any traces of phenol and then resuspended in water at a concentration of $8x10^{10}$ organisms per ml. The suspension of vibrios were sonicated in an ice bath at 80 watts, three times for 10 second intervals. The sonicated samples were aliquoted and stored at -20^0C.

2.3.5.2 Extraction of LPS

LPS from the Ogawa and Inaba serotypes were extracted essentially by the process of Westphal and Jann (1965).

Vibrios suspended in 0.5% phenol-saline at a concentration of $8x10^{10}$ and 90% (w/v) phenol were separately

heated to 65^0C. Equal volumes of each were mixed thoroughly and the mixture was incubated for 15 minutes at 65^0C. The mixture was then cooled to 10^0C in ice and centrifuged at 3000rpm for 45 minutes. The aqueous phase was removed and the phenol phase was reextracted with water. The two aqueous phases were pooled and dialysed against water for 48 hours. The dialysed sample was centrifuged at 105,000xg at 4^0C and the LPS was obtained as a gelatinous precipitate. The precipitate was dissolved in water and stored at 4^0C. This crude LPS preparation was used throughout the study unless stated otherwise.

The yield of LPS was determined by the dry weight of the LPS.

2.3.5.3 Protein estimation of sonicated extract and LPS

2.3.5.3.1 Protein estimation by Bradford's method

Bradford's reagent (Bradford,1976) was prepared fresh before use by dissolving 100mg of Coomassie Brilliant Blue G-250 in 50ml of 95% ethanol. 100ml of 85% w/v phoshporic acid were added to it and the volume was made upto a litre. The solution was filtered before use.

A duplicate series of tubes containing 10, 20, 30, 40 and 50µg BSA per 100µl (from a stock solution of 1mg/ml) were set up. The volume in the test tubes were kept constant at 100µl. 0.3ml of Bradford's reagent was added to each to tube and mixed well and incubated for 10 minutes at room temperature. The optical density of the contents of the tubes were measured at 595nm and a standard curve was drawn.

LPS and sonicated samples of vibrios were treated in a similar fashion and their protein content was determined by reading the concentration from the standard corresponding to their optical densities.

2.3.5.3.2 Protein estimation by the method of Lowry

Solutions

A. 2% $NaCO_3$ in 0.1N NaOH

B. 0.5% $CuSO_4.5H_2O$ in 1% potassium tartarate

C. 1ml of solutin B mixed with 50ml of solution A. This solution was prepared fresh before use.

E. Folin's reagent

Standard BSA solution (1mg/ml)

Procedure

Test tubes were set up in duplicates with 10-100µg of BSA and samples whose protein content was to be determined. The volumes were kept constant at 100µl. 1ml of reagent C was added to each test tube and incubated for 10 ten minutes. 0.1ml of Folin's reagent was then added and mixed well. The tubes were incubated for another 30 minutes and the optical density was measured at 750nm. The standard graph was plotted and the concentrations of unknown samples were obtained from the graph.

2.3.5.4 Polyacrylamide Gel Electrophoresis

Molecular weight determination and blotting of LPS and protein molecules were carried out on denaturing gels. The gels

were prepared and run as described by Laemmli (1970).

The stock solutions used were as described below:

Stock solutions

Acrylamide-bisacrylamide (30:0.8)

30g of acrylamide and 0.8g of bisacrylamide were dissolved in 100ml of water. The solution was then filtered and stored in a dark bottle at 4^0C.

Resolving gel buffer

3M Tris-HCl pH 8.8

Stacking gel buffer

0.5M Tris-HCl pH 6.8

Ammonium persulphate

15% w/v of ammonium persulphate was prepared in water just before use.

Reservoir buffer

The buffer consisted of 0.025M Tris base, 0.192M glycine and 0.1% SDS. The pH was adjusted to 8.3.

Sample buffer

The buffer consisted of 0.0625M Tris base, 2% SDS, 10% glycerol,5% 2-mercapto-ethanol and 0.001% bromophenol blue. The pH was adjusted to 6.8.

Coomassie Blue Stain

The stain was made up by dissolving 1g of Coomassie Blue G-250 in 250ml of methanol and 100ml of acetic acid.

Destain for Coomassie Blue

The destain consisted of 40% methanol, 10% acetic acid in distilled water.

2.3.5.4.1 Preparation of slab gels

Slab gels of varying lengths and concentrations of acrylamide were prepared as stated in the respective sections.

2.3.5.4.2 Electrophoresis of Sonicated Vibrios

Equal volumes of the sonicated suspension (Section 2.3.5.1) and sample buffer were mixed and boiled at 100^0C for 5 minutes. An aliquot of this mixture was loaded on a 15% slab gel 10cm long. The gels were run at 60 mamps till the bromophenol blue had reached the end of the gel.

The gels were stained with Coomassie Blue for 2-4 hours or silver stained. A replica of the gel was used for electrophoretic transfer on nitrocellulose paper (described in Section 2.3.5.10). The Coomassie blue stained gels were destained to remove the backround colour and facilitate the examination of the stained bands.

2.3.5.4.3 Electrophoresis of LPS

Crude and pure (Sigma) LPS were dissolved in water and suspended in equal volumes of sample buffer and boiled at 100^0C for 5 minutes. Appropriate volumes of the mixture were then loaded on 15% or 20% gels.

2.3.5.5 Silver Stain for LPS

Sonicated vibrios or crude LPS extract on gels were silver stained by the method of (Tsai and Frasch 1982) to detect

the LPS. All glassware and plastic containers to be used in this method were thoroughly cleaned and rinsed with double deionised water. Double deionised water was used throughout in this method.

Solutions for Silver Stain

Solution for fixing Gels

40% ethanol and 10% acetic acid in water.

Oxidising Solution

0.7% periodic acid in fixing solution.

Silver Stain

28ml of 0.1N NaOH, 2ml of concentrated ammonia, 5ml of 20% silver nitrate and 115ml of water.

Developer

0.005% citric acid and 100ul of 37% formaldehyde in a litre of water.

Solution for preserving the silver stain

3% acetic acid

Procedure

After the gel electropheresis was complete the gel was placed in the fixing solution overnight.

The following day the gel was incubated in the oxidising solution for 5 minutes with shaking. The gel was then washed for 45 minutes with atleast 5 changes of water. After the last wash the silver stain was added and the gel incubated in it for 10 minutes with vigorous shaking. The stain was discarded and the gel was washed again for 30 minutes with 4-5 changes of water. The gel was then incubated in the developer until the bands

appeared with the desired intensity. The gel was then washed in water and preserved in 3% acetic acid.

2.3.5.6 Resolution of V.cholerae LPS

2.3.5.6.1 Resolution on 20% acrylamide

2-20ng of LPS were loaded on 40-50cm long gels and the samples were electrophoresed at 50mamps until the tracking dye had reached the end of the gel and for further two hours at the same current. The gel was then silver stained.

2.3.5.6.2 Resolution on 15% acrylamide

2-20ng of LPS were loaded on 40-50cm long gels and the samples were electrophoresed at 50mamps until the tracking dye had reached the end of the gel and for further two hours at the same current. The gel was then silver stained.

2.3.5.6.3 Resolution of high mobility band

20-60ng of LPS were loaded on 90cm long gels and electrophoresed at 50mamps for 8 hours and at 20mamps for 16 hours and by then the dye had reached the end of the gel. The current was then increased to 50mamps and the electrophoresis was continued for another 2-3 hours. The gel was then cut in two halves lengthwise and stained for LPS.

2.3.5.7. RNAse digest of crude LPS

Crude LPS from either serotype were digested with 50µg/ml of RNAse in 10mM Tris pH7.2 and 1mM EDTA for 30 minutes at 37^{0}C. After the digestion was complete the RNAse was extracted

from the solution by 90% phenol at 65^{0}C. The LPS remaining in the aqueous phase was dialysed against water for 24 hours before being studied on SDS-PAGE by silver staining.

2.3.5.8 LPS digestion with ∝-mannosidase, β- and ∝-glucosidase

A time-course digest of the LPS with the respective enzymes was carried out to study the presence of any terminal mannose, β- or ∝- glucose residues in the LPS molecule.

500ng of LPS were digested with 10 units of ∝-mannosidase, 10 units of β-glucosidase and 25 units of∝-glucosidase for different lengths of time at 37^{0}C. Equal volume of sample buffer was added to each digested sample and boiled for 5 minutes to stop the enzyme reaction. The samples were then ready for SDS-PAGE analysis.

2.3.5.9 Acid Hydrolysis of LPS

LPS was hydrolysed with 1% acetic acid at 100^{0}C for different lengths of time. The hydrolysed samples were neutralised with 0.1N NaOH and analysed on SDS-PAGE by silver staining. An aliquot of hydrolysed sample was extracted with ether. Ether was removed from the aqueous phase by blowing air and the ether phase was air dried.

2.3.5.10 Electroblotting

Transfer buffer

The buffer contained 25mM Tris-base, 0.19M glycine, 20% v/v methanol and 0.02 % SDS. The pH was adjusted to 8.6.

Proteins and LPS were electrophoretically transferred onto nitrocellulose paper to enable binding studies with antibodies. The transfer was carried out essentially by the method of Batteiger (1981). Briefly, a plastic grid followed by a buffer-soaked Scotch-Brite pad and then a soaked filter paper was placed under the piece of gel to be electrophoresed. A nitrocellulose paper also soaked in buffer was then carefully placed on the wet gel so as to exclude any air bubble between the gel and the nitrocellulose paper. This was followed by a wet filter paper and another pad and a plastic grid. The assembly was immersed with the nitrocellulose paper closest to the anode in a BIO-RAD tank for electroblotting filled with the transfer buffer. The electrophoretic transfer was carried on at 350-400mamps for 2.5-3 hours.

PRODUCTION AND PRELIMINARY CHARACTERISATION OF MONOCLONAL ANTIBODIES TO V.CHOLERAE

3.1 Experimental approach

The rat system for the production of monoclonal antibodies was selected in this study. The rat cell lines Y3/Ag.1.2.3 and YB2/0 and the DA ratswere readily available.

Heat killed and phenol preserved vibrios were injected into animals. Heat killed vibrios were used as they corresponded most closely to the routine vaccines.

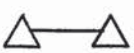
3.2 Specificity of Rat Anti-sera against V.cholerae

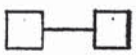
Normal rat serum and anti-Ogawa and anti-Inaba sera obtained from several rats were tested against Ogawa and Inaba vibrios on ELISA (Section 2.3.3.1). The optimum number of vibrios coated on ELISA plates were determined by performing a chequer-board ELISA with anti-Ogawa and anti-Inaba antisera and normal rat serum. Figure 3.1 illustrates the reaction of antisera and normal rat sera against various amounts of vibrios. Antisera exhibited a very strong reaction against 1.2×10^6 vibrios and the reaction of normal rat sera was low, so 10^6 vibrios were selected as the optimum concentration of vibrios for future assays on

Fig.3.1 Chequer-board ELISA

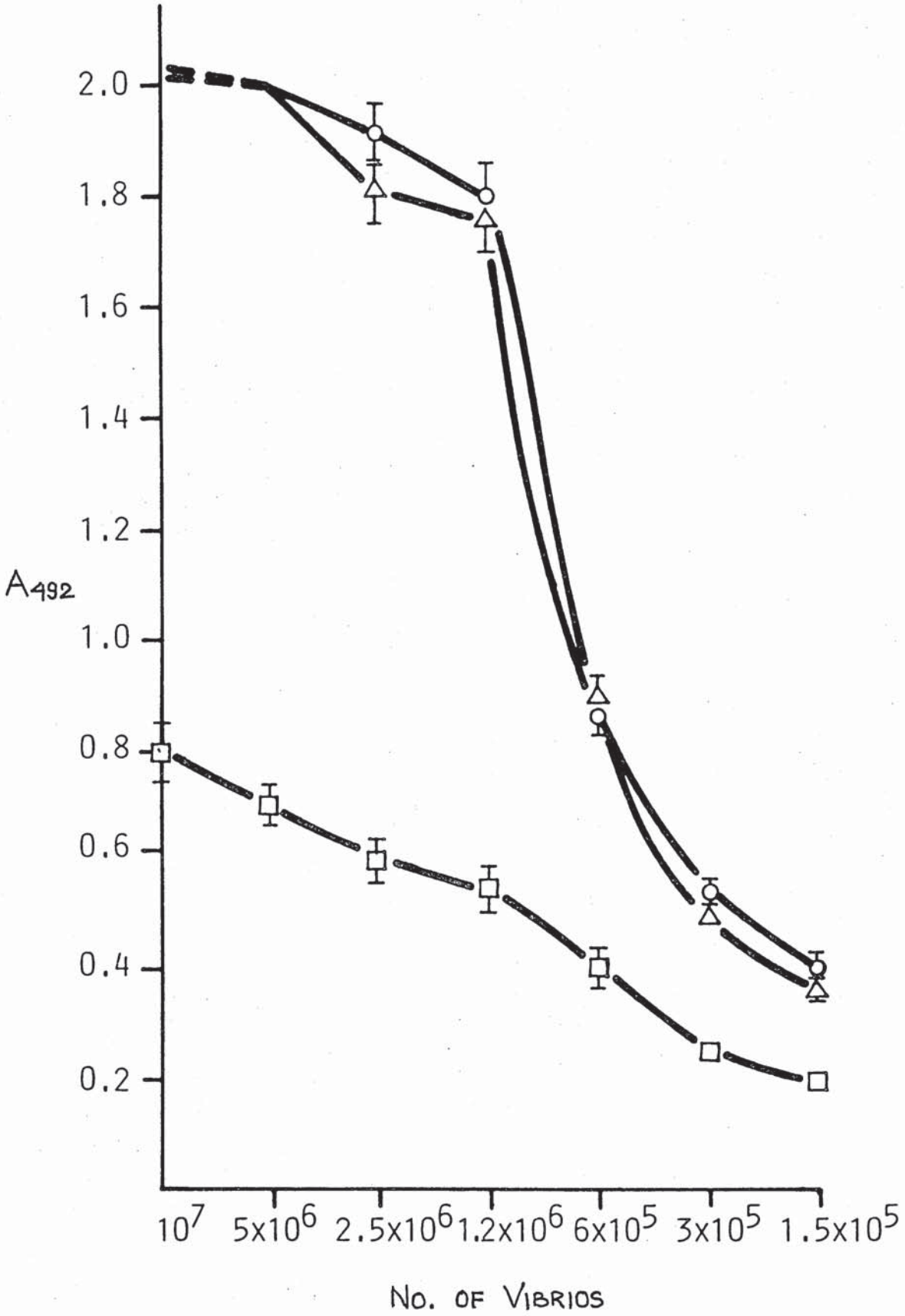
Various amounts of vibrios were coated on ELISA plate and sera from immunised and unimmunised rats were reacted with them. The assay was repeated three times. The bars represent the deviation between three assays.

 Reaction of anti-Ogawa antiserum against Ogawa vibrios

 Reaction of anti-Inaba antiserum against Inaba vibrios

 Average reaction of unimmunised rat serum against Ogawa and Inaba vibrios

Background reaction was below 0.1 O.D.


2.0
1.8
1.6
1.4
1.2
A492
1.0
0.8
0.6
0.4
0.2
10^7
5x10^6
2.5x10^6
1.2x10^6
6x10^5
3x10^5
1.5x10^5
No. of Vibrios

ELISA. The ELISA reactions of antisera and normal sera on two serotypes of V. cholerae are illustrated in Figure 3.2. Normal rat sera had little activity against the two serotypes of V. cholerae. Anti-Ogawa antisera reacted with both Ogawa and Inaba and had high titres against both serotypes. Anti-Inaba antisera also reacted strongly with Inaba and Ogawa vibrios. However, the reaction of each type of antisera was stronger against the immunising serotype, this is in agreement with the results obtained by Cryz et al, (1982).

3.3 Presence of A,B and C Antigens on V.cholerae

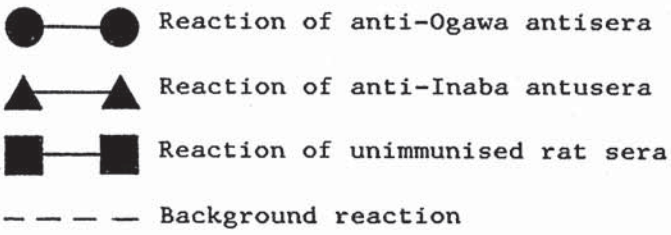
Among the heat stable lipopolysaccharide antigens of V. cholerae, serogroup O:1, the group-specific antigen A, is shared by Inaba and Ogawa serotypes. The type-specific antigen B is Ogawa specific and C is Inaba specific (Kauffmann, 1950). The results of the activity of the polyclonal antisera (Section 3.2) are in agreement with the conventional serological classification of V. cholerae. The anti-Ogawa sera may be presumed to react with the A and B antigens, hence the cross-reaction with Inaba vibrios. Similarly the anti-Inaba sera may be presumed to cross-react with the A antigens on Ogawa vibrios.

3.4 Myeloma Lines Used as Fusion Partners

3.4.1 Y3/Ag.1.2.3 Myeloma Line

Y3 cell line was cultured in complete medium containing 10% FCS.The cells could be grown in spinner or Nunc T-75 culture

Fig.3.2 a ELISA of antisera and unimmunised rat sera against Inaba vibrios



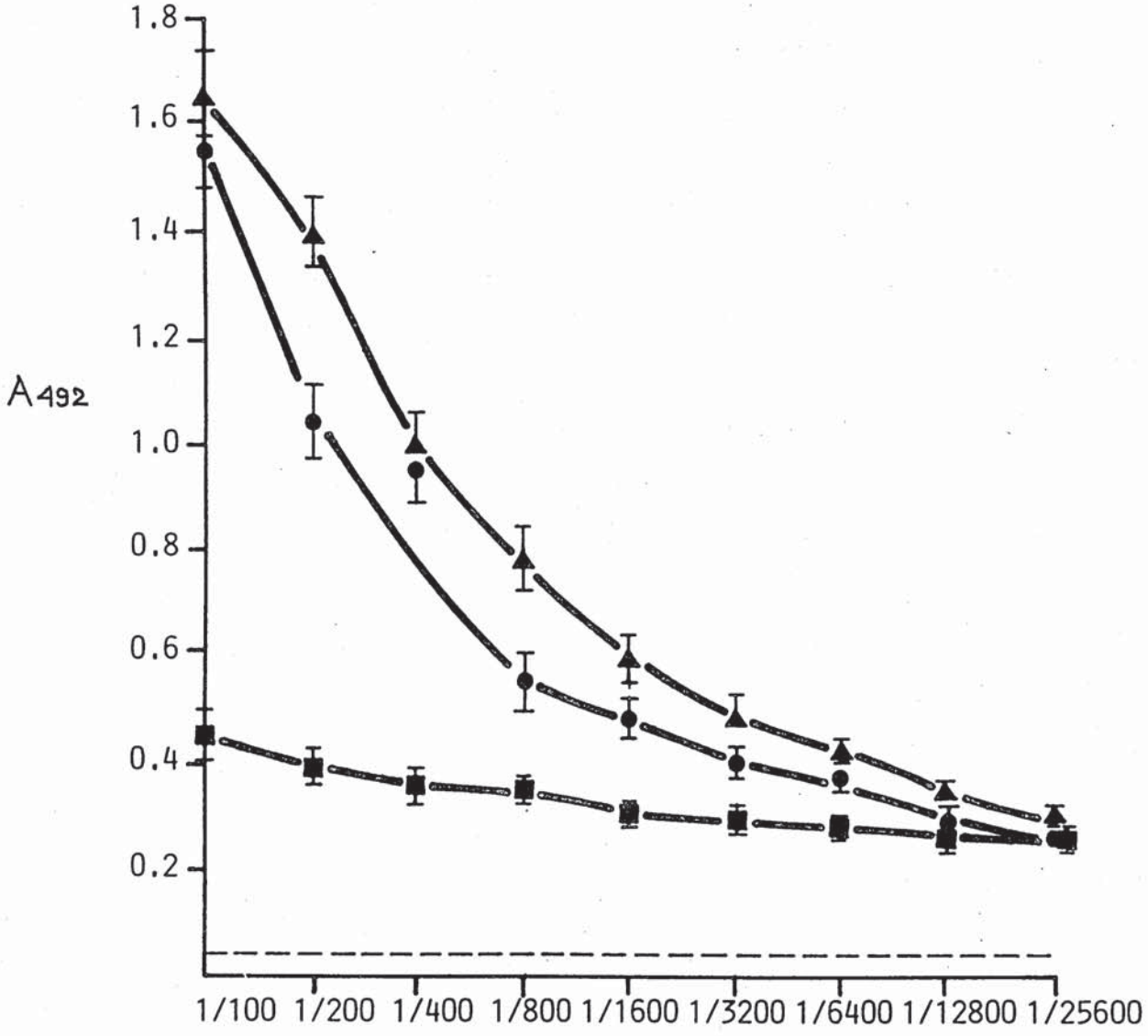
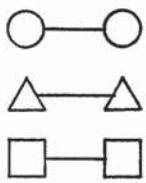

1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
A492
1/100 1/200 1/400 1/800 1/1600 1/3200 1/6400 1/12800 1/25600
ANTIBODY DILUTION

Fig.3.2 b ELISA of antisera and unimmunised rat sera against Ogawa vibrios

Antisera from ten different rats were tested and the mean values were plotted, the bars indicate the variation between each assay.

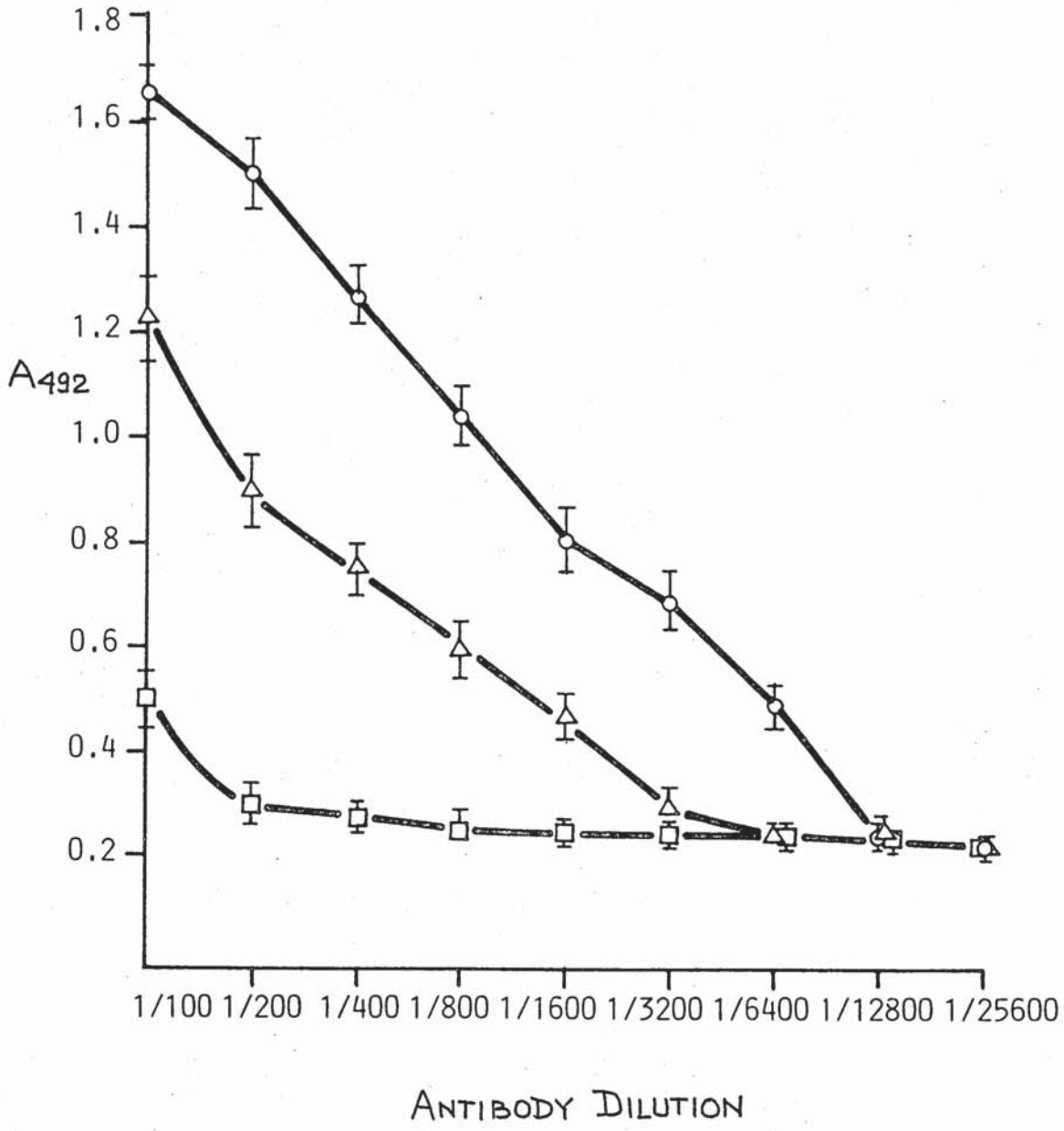


Reaction of anti-Ogawa antisera

Reaction of anti-Inaba antisera

Reaction of unimmunised rat sera

Background reaction was below 0.1 O.D.


1.8
1.6
1.4
1.2
A492
1.0
0.8
0.6
0.4
0.2
1/100 1/200 1/400 1/800 1/1600 1/3200 1/6400 1/12800 1/25600
ANTIBODY DILUTION

flasks. The doubling time for the cells was 18-20 hours.

3.4.2 YB2/0 Myeloma Line

YB2/0 cells were cultured in complete medium containing 10% FCS. The doubling time of the cells were about 30 hours.

3.5 Hybridomas Raised From Rat Myeloma Lines

3.5.1 Hybridomas derived from Y3/Ag.1.2.3 Myeloma Line

Ogawa or Inaba immunised rat spleen cells were fused with Y3/Ag.1.2.3 cells obtained from a spinner culture or a T-75 culture flask. The Y3/Ag 1.2.3 cells from a spinner culture were superior fusion partners to Flask grown cells. When they were cultured in a Nunc flask, a high proportion of cells stuck to the surface of the flask and had to be shaken off before the fusion. Spleen cells fused with Y3/Ag.1.2.3 from a spinner culture gave a higher percentage of hybridomas than cells fused with Y3/Ag.1.2.3 grown in a T-75 flask (Table 3.1). The average time period for the hybridomas to be visible after a fusion was 8-15 days. Hybridomas derived from flask cultured Y3 cells usually took longer to emerge.

The emerging hybridomas were screened for secretion of antibodies reactive with Ogawa and Inaba on ELISA on the third or fourth week after fusion. The positive clones were expanded from the 2ml wells into 25ml culture flasks. The cells were then subcloned (Section 2.4.8) and some were kept frozen in liquid nitrogen. Frozen primary clones could subsequently be recovered and cloned if necessary.

Fusion No.	Culture of Y3	Percentage of wells with hybridomas	Percentage of positive hybrids
1	T-5 Flask	2	100
2	T-5 Flask	6	50
3	T-5 Flask	20	60
4	Spinner Flask	80	60
5	Spinner Flask	65	-
6	Spinner Flask	45	-

Table 3.1 Effect of culture conditions of Y3 cells on yield of hybridomas

3.5.2 Hybridomas derived from fusion with YB2/0 Myeloma Line

Spleen cells from a rat hyper-immunised with Inaba vibrios were fused with YB2/0 cells grown in a spinner culture. 10-15 days after the fusion 70 out of 90 wells in the fusion plates had hybridoma growth in them. The hybridomas were then screened on ELISA. 50% of the clones secreted antibodies against Inaba vibrios. The cells from the positive wells were expanded in culture flasks. The cells grew at a very slow rate in the flask and most of them eventually died. This difficulty in expansion rendered the cells unsuitable for antibody production and the YB2/0 line was not pursued further.

3.5.3 Subclones Screened on ELISA

10-12 days after subcloning 90-95% of the wells subcloned at 0.5 cells per well had cells growing in them. About 50% of the wells subcloned at 0.25 cells per well had cell growth, presumably due to slight inaccuracies in cell counting. The supernatants from the wells were assayed on ELISA. Almost every subclone secreted antibodies similar to that of the parent clone in the original fusion plate. This confirms the reports from others on the early stability of the rat system (Clark et al, 1982). Subcloning was performed three times at 0.25 cells/well before a hybridoma was presumed to secrete monoclonal antibodies.

3.6 Specificity of Monoclonal Antibodies

Monoclonal antibodies raised against the Ogawa serotype were O4A6, O3D1 and O4D2, each derived from a separate fusion. The monoclonal antibodies raised against Inaba serotype were I1A1,I4B1 and I4C3 all derived from the same fusion. The six established clones were reacted on ELISA with a panel of distantly related gram-negative bacteria to detect any cross-reactions between serotypes of O1 group and other unrelated O-antigens.

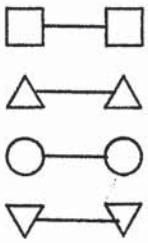
Figure 3.3a shows the reaction of O4A6, O3D1 and O4D2 against Ogawa, Inaba, *E. coli*, *B. abortus* and *B. melitensis* on ELISA. The monoclonal antibodies do not react with the Inaba serotype. None of the three monoclonal antibodies bound to any of the other gram-negative bacteria. Culture supernatants of O4A6 and O3D1 had a titre lower than 1 in 512 and O4D2 had a titre of 1 in 512 against the Ogawa serotype.

The reaction of I1A1, I4B1 and I4C3 is demonstrated in Fig.3.3b. The monoclonal antibodies reacted very strongly with Inaba vibrios. None of the three MCAbs appeared to react with the non-O1 group of antigens. The titre of I1A1 was 1 in 256 and that of I4B1 and I4C3 were less than 1 in 512 against Inaba. It should be noted that while all three of these antibodies appear to react at background level with Ogawa vibrios, the background is slightly higher than that obtained with the Ogawa specific monoclonals on the Inaba vibrios.

MCAbs raised against A, B and C antigens by Gustafsson and

Fig.3.3 a Titration of rat MCAbs on various gram-negative bacteria

Each MCAb was titred on 10^6 bacteria. The values plotted are a mean of five asays.



Reaction of O4A6 against Ogawa

Reaction of O3D1 against Ogawa

Reaction of O4D2 against Ogawa

Average reaction of O4A6, O4D2 and O3D1 on Inaba, *B. abortus*, *B. melitensis* and *E. coli* ML 308

Background reaction

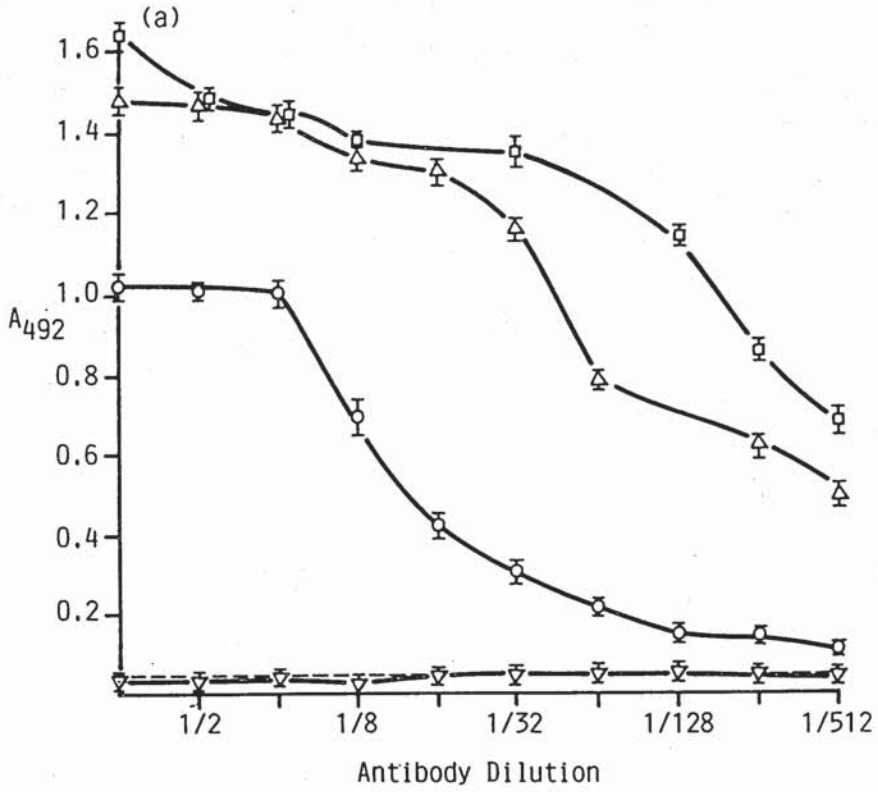
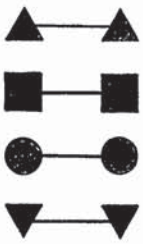

(a)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
A492
1/2
1/8
1/32
1/128
1/512
Antibody Dilution

Fig.3.3 b Titration of rat MCAbs on various gram-negative bacteria

Each MCAb was titred on 10^6 bacteria. The values plotted are a mean of five asays.



Reaction of I1A1 against Inaba

Reaction of I4B1 against Inaba

Reaction of I4C3 against Inaba

Average reaction of I1A1, I4B1 and I4C3 on Inaba, *B. abortus*, *B. melitensis* and *E. coli* ML 308

Background reaction

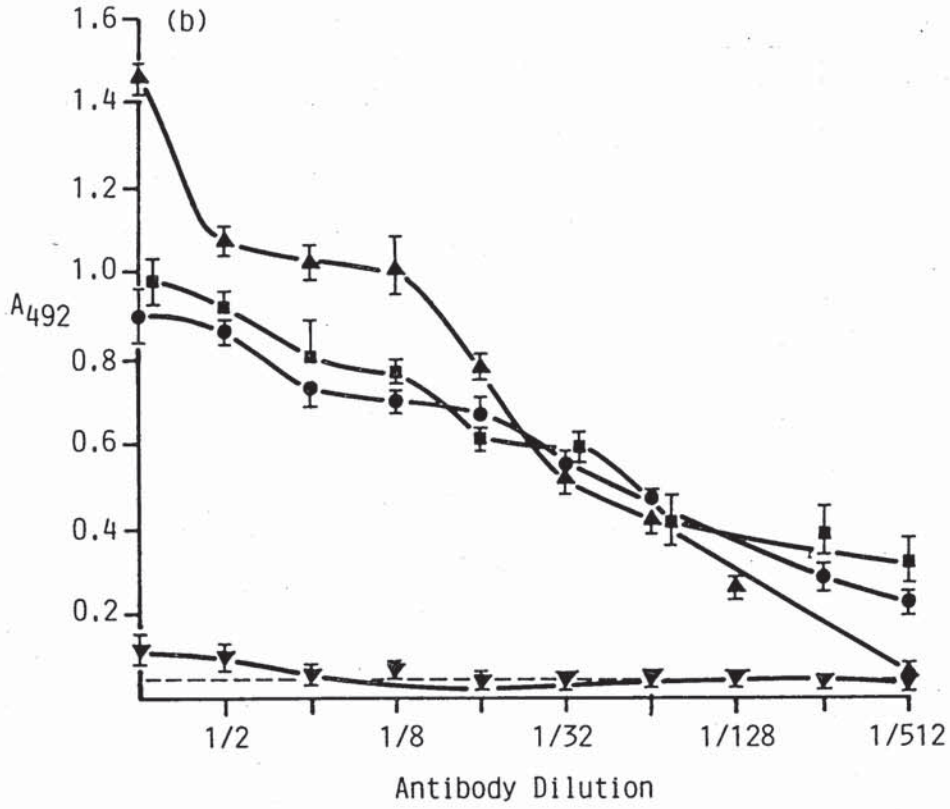
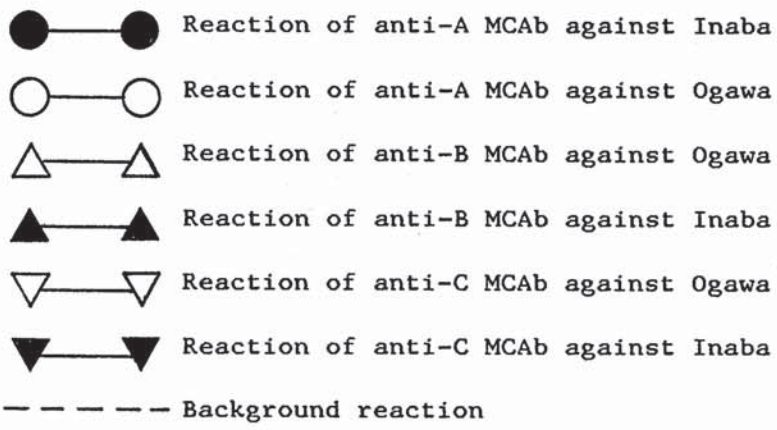
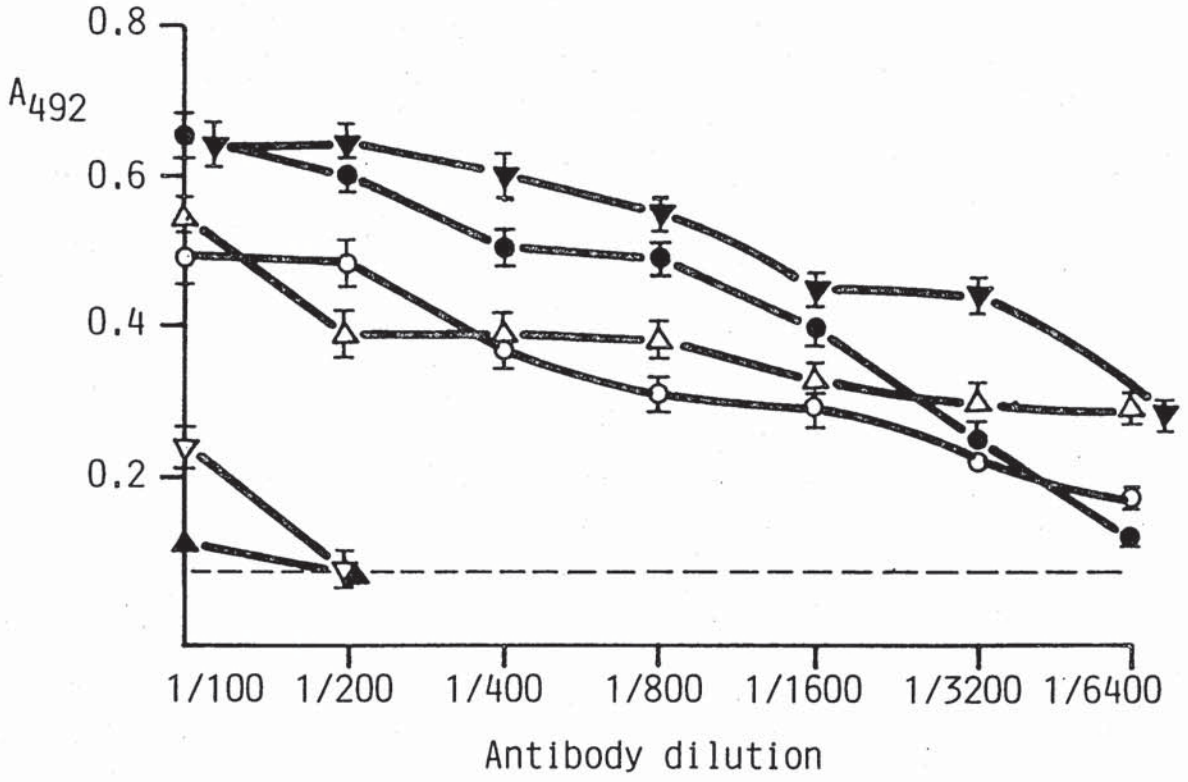

(b)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
A492
1/2
1/8
1/32
1/128
1/512
Antibody Dilution

Fig.3.3 c Titration of anti-V. cholerae mouse MCAbs

Mouse MCAbs as ascites were obtained from Sweden (Section 3.6). The antibodies were titred on 10^6 bacteria. The values plotted are a mean of two asays.




0.8
A_{492}
0.6
0.4
0.2
1/100
1/200
1/400
1/800
1/1600
1/3200
1/6400
Antibody dilution

Holme (1983) were received in exchange and tested on ELISA as with the other MCAbs with the exception of the blocking buffer. The MCAbs received as ascitic fluid reacted with BSA and hence the ELISA plates were blocked with 5% normal goat serum. On doing so, the MCAbs exhibited their respective specifities (Fig.3.3c).

Consequently, primary screening of the MCAbs established in this study detected three monoclonal antibodies apparently specific to the B antigen of classical serology and three apparently specific to the C.

3.7 Class and Subclass Analysis of Monoclonal Antibodies

3.7.1 Immunoglobulin Class and Subclass determination by Ouchterlony

The method of Ouchterlony (Ouchterlony and Nilsson, 1978) involved precipitation of antibodies by a polyclonal antiserum in agar. Anti-rat IgG, IgM, IgG1, IgG2a, IgG2b and IgG2c were reacted with rat serum. Each of the anti-rat immunoglobulin class and subclass antisera reacted with the various subclasses of the immunoglobulins present in the rat serum. The major immunoglobulin component of the rat serum was IgG. The distribution of the subclasses of IgG in the serum were in the decreasing order of IgG1, IgG2a, IgG2c and IgG2b as judged by the intensity of the precipitin lines obtained with the reagents used (Figure 3.4a).

Immunoglobulins of all six hybridomas were precipitated only by anti-rat IgG2b (Figure 3.4b). It was necessary to

Fig.3.4 a Ouchterlony of rat antisera

Polyclonal antiserum against rat IgG1, IgG2a, IgG2b and IgG2c were added in the centre wells of 1, 2, 3 and 4 respectively. Rat antiserum was added in doubling dilutions in the peripheral wells.

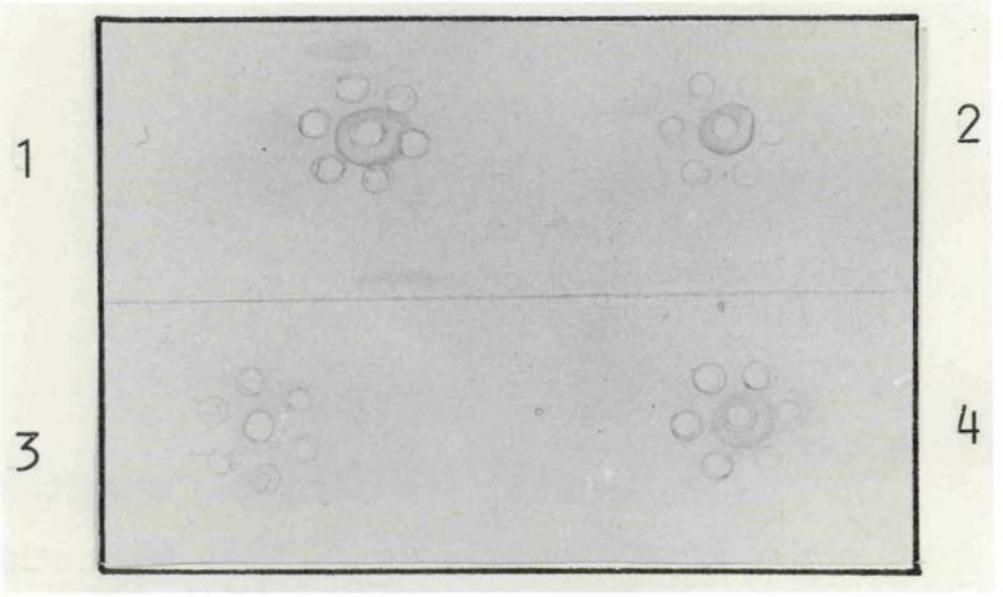
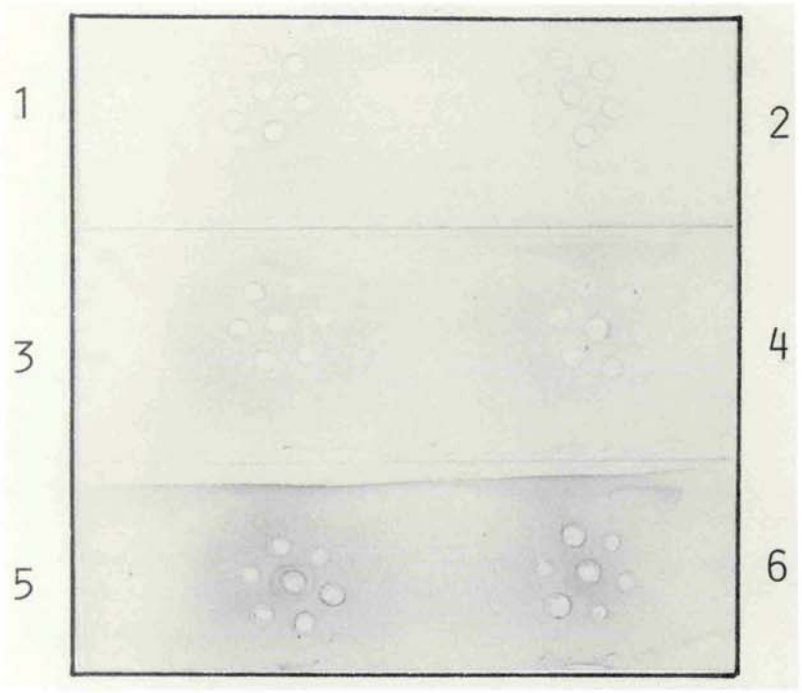

1
2
3
4

Fig.3.4 b Ouchterlony of rat MCAbs

Polyclonal antiserum against rat IgG, IgM, IgG1, IgG2a, IgG2b and IgG2c were added in the centre wells of 1, 2, 3, 4, 5 and 6 respectively. Rat MCAb 04A6 was added in doubling dilutions in the peripheral wells.


1
2
3
4
5
6

concentrate the tissue culture supernatant 10 fold with ammonium sulphate (Section 2.3.4.3) to obtain any precipitation.

Attempts to confirm the subclass allocation by ELISA did not yield a clear picture because of the nature of the commercial antisubclass reagents. The four anti-rat Ig subclass reagents were available as antisera from three species of animals (Miles), hence comparison of data was inconvenient. Moreover, the reagents reacted with light chains.

It is of interest to note that all six monoclonal antibodies are of the same subclass. Mouse monoclonal antibodies reactive with bacterial lipopolysaccharides and carbohydrates in general tend to be either IgMs or of the IgG3 subclass which is a minor serum component (Section 6.4) and the data suggest that IgG2b may be the equivalent subclass in the rat.

3.7.2 SDS-PAGE Studies of MCAbs Precipitated with Second Antibody

The immunoglobulins secreted by the established hybridomas were analysed on 10% SDS-PAGE by internal labelling of the hybridomas with ^{35}S-methionine (Section 2.3.4.1). The Y3/Ag.1.2.3 cell line and mouse myeloma line, P3/X63 Ag.8 (Kohler and Milstein, 1975) were also labelled for comparison.

The second antibodies used were goat anti-rat IgG and goat anti-rat IgM. The precipitating antiserum was donkey anti-goat. Both anti-rat IgG and anti-rat IgM precipitated the Y3/Ag 1.2.3 kappa light chain and the monoclonal antibodies, presumably due to reaction with their light chain as there were

no heavy chains apparent on the gels or fluorographs (Figure3.5b). All the monoclonal antibodies made a heavy chain of molecular weight 50-51,000 and all but O4A6 made two light chains(Figure 3.5a). The two light chains may either represent the Y3 light chain together with that of the monoclonal antibody or differential glycosylation of a single light chain (Kohler and Milstein, 1976).

Fig.3.5 a SDS-PAGE of MCAbs precipitated with polyclonal antisera

MCAbs were internally labelled and precipitated as described in Section 2.3.4.1.

Lane 1 O4A6 precipitated with anti-rat IgG

Lane 2 O4A6 precipitated with normal goat serum

Lane 3 O3D1 precipitated with anti-rat IgG

Lane 4 O4D2 precipitated with anti-rat IgG

Lane 5 P3 precipitated with anti-mouse IgG

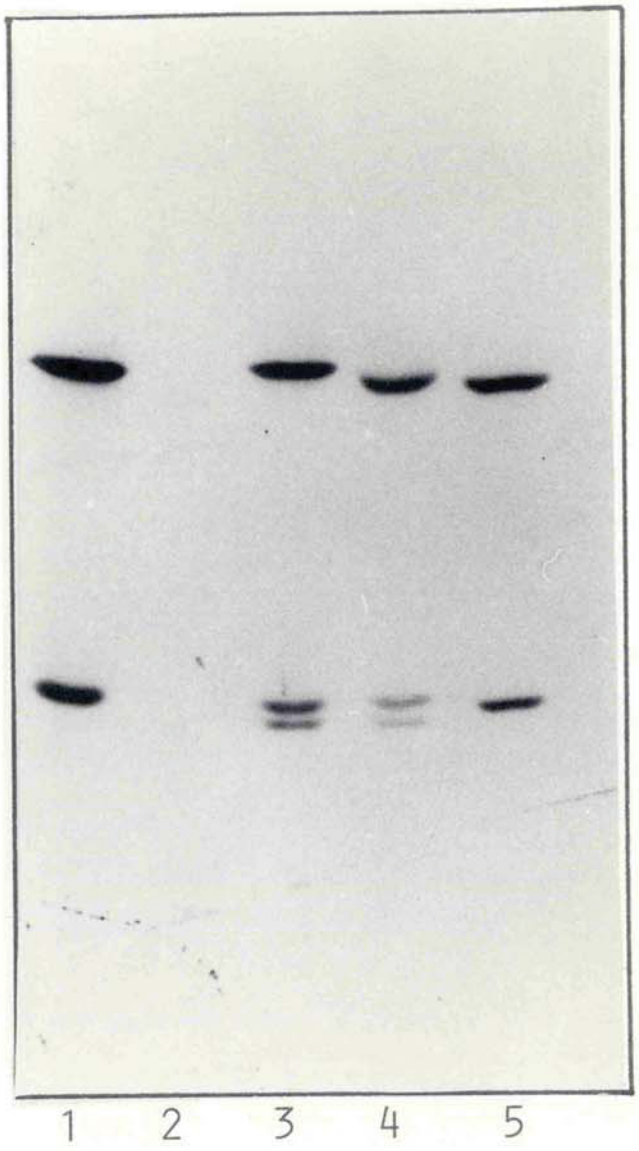

1
2
3
4
5

Fig.3.5 b SDS-PAGE of MCAbs precipitated with polyclonal antisera

MCAbs were internally labelled and precipitated as described in Section 2.3.4.1.

Lane 1 Y3 precipitated with anti-rat IgG

Lane 2 Y3 precipitated with anti-rat IgM

Lane 3 I1A1 precipitated with anti-rat IgG

Lane 4 I1A1 precipitated with anti-rat IgM

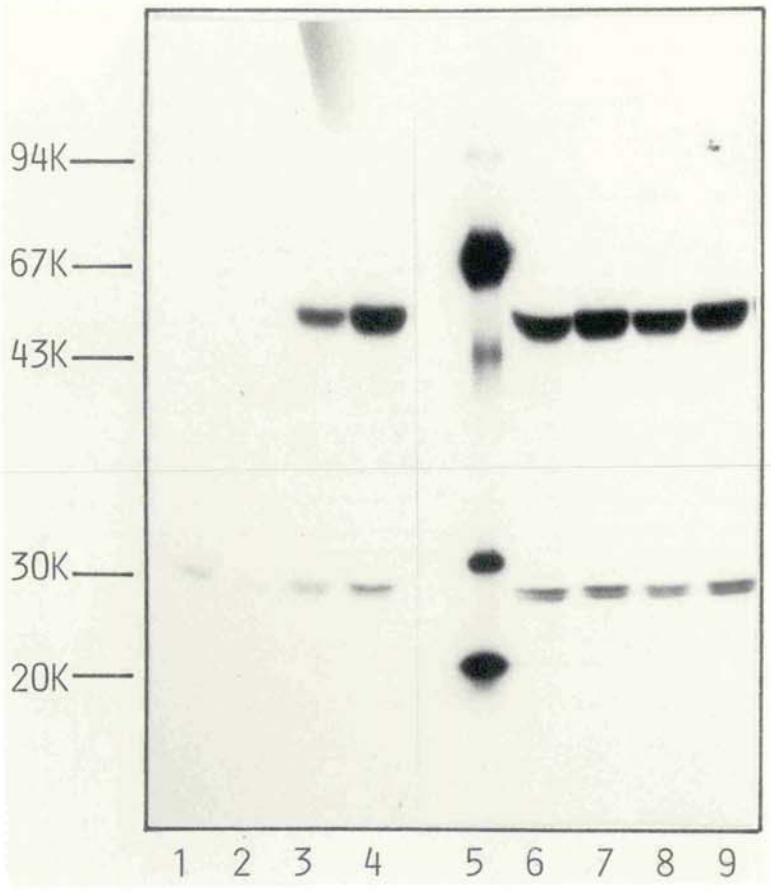
Lane 5 Radioiodinated Protein molecular weight markers

Lane 6 I4C3 precipitated with anti-rat IgG

Lane 7 I4C3 precipitated with anti-rat IgM

Lane 8 I4B1 precipitated with anti-rat IgG

Lane 9 I4B1 precipitated with anti-rat IgM


94K
67K
43K
30K
20K
1 2 3 4 5 6 7 8 9

ANALYSIS OF V. CHOLERAE LPS ON SDS-PAGE

4.1 Outer membrane of V. cholerae

The V. cholerae outer membrane consists of lipopolysaccharide and protein. The LPS is believed to be responsible for the major host immune response (Levine et al, 1979; Majumdar and Ghose, 1981; Majumdar et al, 1981). Low molecular weight outer membrane proteins have also been reported to be immunogenic (Kabir, 1980; Stevenson et al, 1985). The reaction of the six MCAbs raised in this study with these components was investigated by Western blots of sonicated vibrios and LPS extracted from the vibrios.

4.2 Sonicated V. cholerae organisms

The protein content of the sonicated vibrios was determined by both the method of Lowry et al (1951) and Bradford (1976). $2x10^{11}$ sonicated vibrios per ml had a protein content of 12.5 mg per ml by the Lowry method and 4.3 mg per ml by the Bradford method of protein estimation.

4.3 Extraction of LPS from the vibrios

V. cholerae LPS was extracted by the hot aqueous-phenol method described in Section 2.3.5.2. The aqueous phase was centrifuged

to obtain the gelatinous precipitate of LPS. The crude LPS thus obtained was not purified any further. The protein contamination in the LPS was 1.2 mg/ml by the Lowry method and 0.2 mg/ml by Bradford method of protein estimation.

Carbohydrate components of LPS of some gram-negative bacteria have been reported to absorb light at specific wavelengths (Afzal et al, 1984). Crude LPS obtained from the serotypes of V. cholerae were scanned over wavelengths 220-700nm to detect any absorption. The LPS absorbed to a small extent at wavelengths of 280-260nm (Fig.4.1) which may have been contributed by the contaminating proteins and nucleic acids but could also be accounted for by light scattering from such a large molecule. The LPS did not otherwise have any characteristic absorption at any wavelength examined. Ogawa and Inaba LPS gave identical results.

4.3.1 Storage of LPS

Crude LPS obtained from heat killed vibrios were dissolved in distilled water and stored at $4^{0}C$ at a concentration of 1µg/ml. No visible aggregation was observed at these conditions of storage for a period of about three months. Aggregation was observed in samples stored for longer than three months.

4.4 V.cholerae proteins estimated by the Lowry's and the Bradford's methods

Many substances are known to interfere with the Lowry's method of protein estimation (Peterson, 1979). The extensive

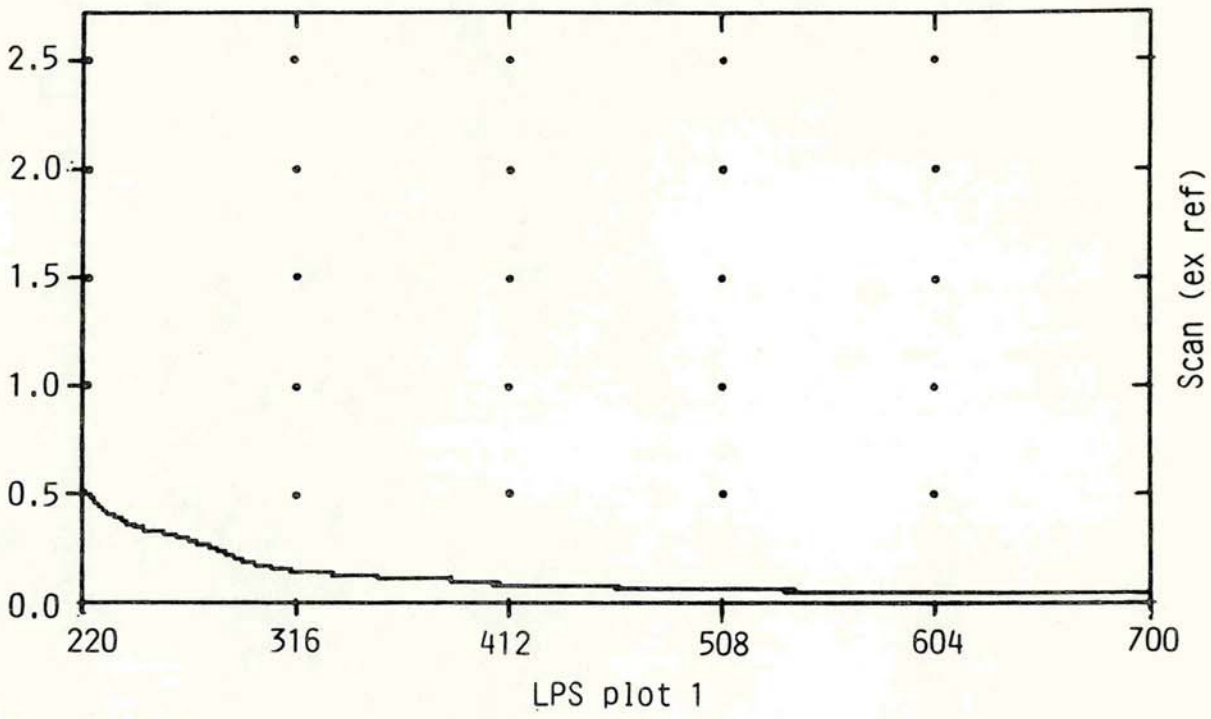


Fig. 4.1 Absorption plot of LPS

LPS from Ogawa serotype at a concentration of 1µg/ml was scanned over wavelengths 220nm to 700nm

list of substances reported by the author to interfere with the assay include lipids and glucosamines, both of which are present in high amounts in the V. cholerae LPS (Raziuddin, 1980). The interference of these two substances may have been the reason for the high values of protein obtained in the sonicated vibrios and the crude LPS by the Lowry's method of estimation. The Bradford's assay has not been reported to be affected by non-protein substances. The protein content as obtained by the Bradford's method of estimation has been taken as the value of protein in the sonicated and the LPS samples.

4.5 Silver stain of sonicated vibrios and LPS

10 µg of protein from the sonicated samples and 15 ng of LPS from each serotype were electrophoresed on 15% polyacrylamide gels (Sections 2.3.5.4.2 and 2.3.5.4.3) and silver stained as described in Section 2.3.5.5. In the sonicated samples of the vibrios a prominent streak (Fig.4.2a) or bulge (Fig.4.2b) was stained at the region of M_r 30K . A major band at about M_r 14K and two other bands in the region of M_r 16K were present along with numerous other faintly stained bands. The pattern of bands for both serotypes were identical.

The LPS preparations from the Inaba 35 A3 and Ogawa NIH 41 serotypes consisted of two major band types, a slow moving band of M_r 30K and a fast moving band of M_r 14K. The low molecular weight band formed a doublet. The Inaba 569B LPS obtained from Sigma showed bands in addition to that observed with Ogawa NIH 41 and Inaba 35 A3. However, the two most

Fig.4.2 a Silver stain of sonicated vibrios

Sonicated vibrios were electrophoresed on 15% acrylamide gel, 10cm long.

Lane 1 Protein molecular weight markers (Pharmacia)

Lane 2 10µg protein from sonicated Ogawa vibrios

Lane 3 10µg protein from sonicated Inaba vibrios

Lane 4 5µg protein from sonicated Ogawa vibrios

Lane 5 5µg protein from sonicated Inaba vibrios

Lane 6 20ng of Inaba 569 B LPS

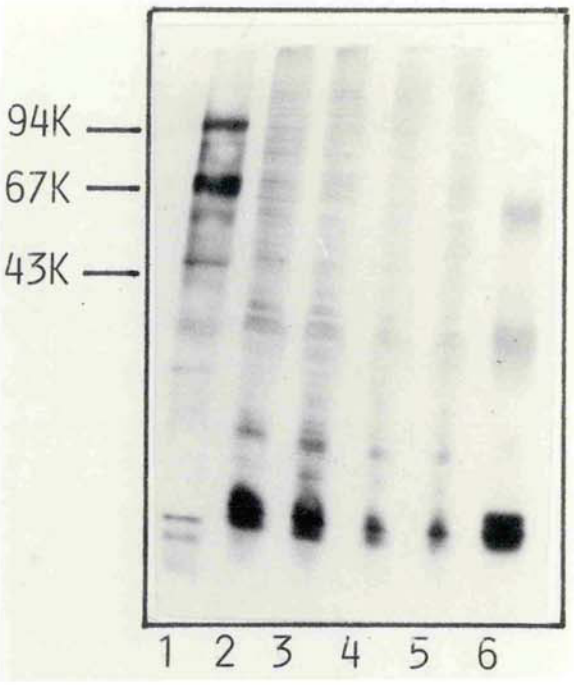

94K
67K
43K
1 2 3 4 5 6

Fig.4.2 b Silver stain of sonicated vibrios and LPS

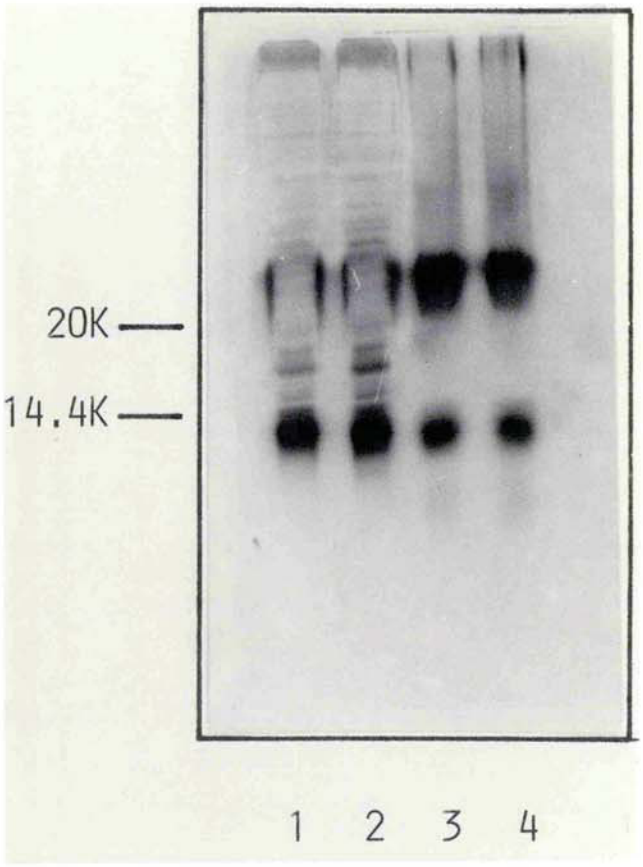
Sonicated vibrios and crude LPS from Ogawa NIH 41 and Inaba 35 A3 were electrophoresed on 15% acrylamide gel, 10cm long.

Lane 1 10μg protein from Ogawa sonicated vibrios

Lane 2 10μg protein from Inaba sonicated vibrios

Lane 3 15ng of Ogawa LPS

Lane 4 15ng of Inaba LPS


20K
14.4K
1
2
3
4

prominent bands coincided with the two bands of Ogawa NIH 41 and Inaba 35 A3.

4.6 V. cholerae LPS on SDS-PAGE

The bands obtained with the crude LPS of both serotypes corresponded with the broad streak of M_r 30K and the doublet of M_r 14K in the sonicated samples (Fig. 4.2). This indicated that the LPS constituted a major portion of the vibrio outer membrane.

The crude LPS on electrophoresis formed a broad band and in the presence of proteins most frequently it streaked at the sides of the track (Fig.4.2b). Typical smooth LPS molecules from other gram negative bacteria separate on electrophoresis to give three components in the complex molecule. The fastest moving band is that of the lipid A. The core migrates just behind the lipid A and it is followed by several O-side chains in the order of increasing molecular weight giving a "ladder" effect (Palva and Makela, 1980; Goldman and Leive, 1980). In the Ogawa NIH 41 and Inaba 35 A3 LPS the fast moving doublet may, by analogy, be tentatively identified as the lipid A and the core. The putative O-side chain did not separate into a "ladder" of units differing by increments in molecular weight but gave a diffuse band. This indicated an apparent lack of size differences in the side chains of the V. cholerae LPS. The Inaba 569B LPS exhibited more than two diffused bands, presumably each one represents O-chains of the particular strain. However, the diffused nature of the slow moving band indicated some heterogeneity which could not be resolved under the conditions employed.

4.7 Coomassie blue stain of sonicated vibrios

10µg of protein from the sonicated samples of the vibrios were electrophoresed on a 15% acrylamide gel and stained for proteins with coomassie blue. Numerous bands in the region of M_r 70-30K were stained but there was comparatively little staining below M_r 30K (Fig. 4.2 c).

4.8 Western blot of sonicated vibrios and crude LPS

LPS and the sonicated vibrios were electrophoresed and blotted as described by (Batteiger, 1982) (Section 2.3.5.10). The blot of the sonicated vibrios was stained with amido black (Fig. 4.3a and b). This reagent does not stain LPS. The LPS was immunoblotted with MCAbs 04A6, I1A1 and I4B1 and detected with a second rabbit anti-rat antibody crosslinked to horseradish peroxidase (Section 2.3.4.5.1).

The MCAbs 04A6 and I1A1 reacted with either the Ogawa or the Inaba serotype respectively (Fig.4.3a). I4B1 reacted with LPS of not only the homologous strain but also with the Inaba 569B (Fig.4.3b), suggesting that the LPS of the two strains are antigenically similar. The four other MCAbs also reacted only with the serotypes originally assigned to them by the ELISA assay (Section 3.6). All six MCAbs reacted only with the slow moving band or bands tentatively identified as the O-antigen, and not the faster moving putative core of the LPS. They did not react with any of the protein antigens in the sonicated samples.

Fig.4.2 c Coomassie blue stain of sonicated vibrios

Sonicated vibrios were electrophoresed on a 15% acrylamide gel, 10cm long.

Lane 1 Protein molecular weight markers (Pharmacia)

Lane 2 10µg protein from Ogawa sonicated vibrios

Lane 3 10µg protein from Inaba sonicated vibrios

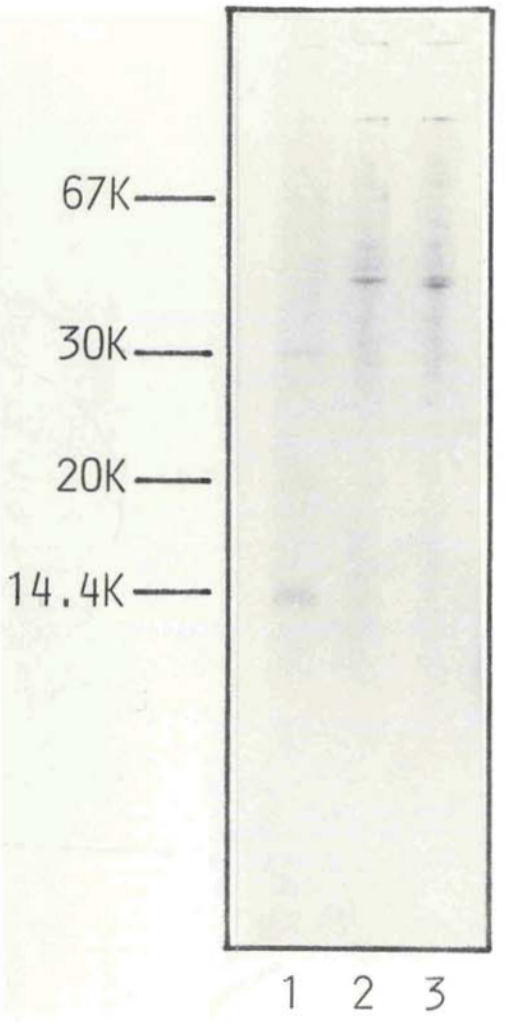

67K
30K
20K
14.4K
1
2
3

Fig. 4.3 a Western blot of sonicated vibrios and LPS

Sonicated vibrios and crude LPS were electrophoresed on 15% acrylamide gel, 10cm long. The gel was electrophoretically blotted onto a nitrocellulose paper and the blots were either stained with amido black or immunostained and developed by enzyme detection method (Section 2.3.4.5.1).

Lanes 1-3 were stained with amido black

Lanes 4-7 were reacted with MCAb I1A1

Lanes 8-11 were reacted with MCAb O4A6

Lane 1 10µg protein from Ogawa sonicated vibrios

Lane 2 10µg protein from Inaba sonicated vibrios

Lane 3 Standard protein molecular weight markers

Lanes 4 and 8 10µg protein from Ogawa sonicated vibrios

Lanes 5 and 9 10µg protein from Inaba sonicated vibrios

Lanes 6 and 10 15ng of Ogawa LPS

Lanes 7 and 11 15ng of Inaba LPS

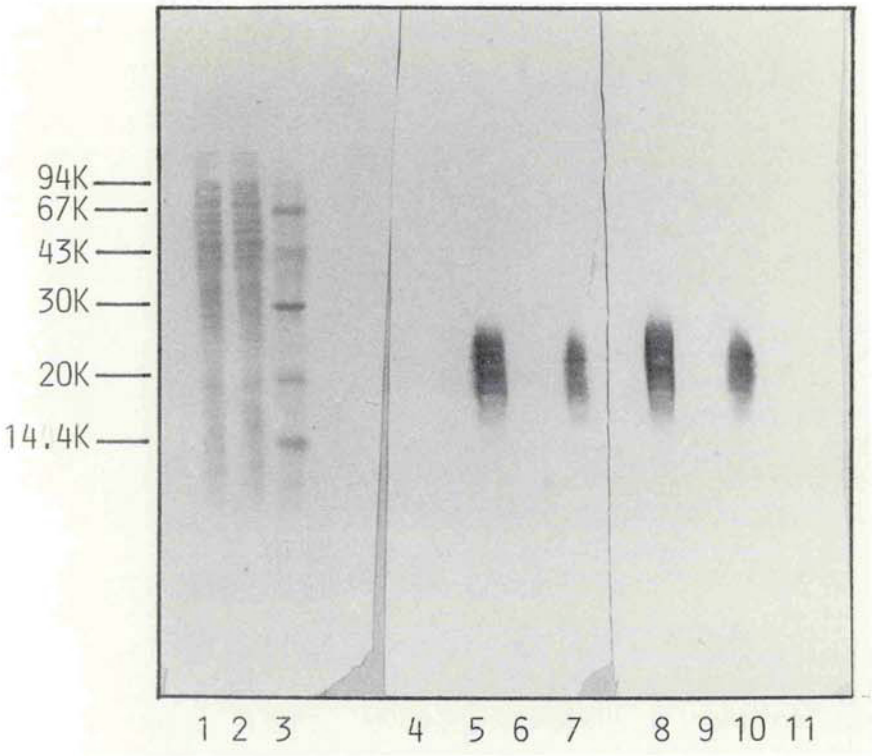

94K
67K
43K
30K
20K
14.4K
1 2 3 4 5 6 7 8 9 10 11

Fig.4.3 b Western blot of sonicated vibrios and LPS

Samples were electrophoresed on 15% acrylamide gel, 10cm long and then electroblotted onto a nitrocellulose paper. Part of the blot was stained with amido black and the other part was immunostained (Section 2.3.4.5.1).

Lanes 1-4 were stained with amido black

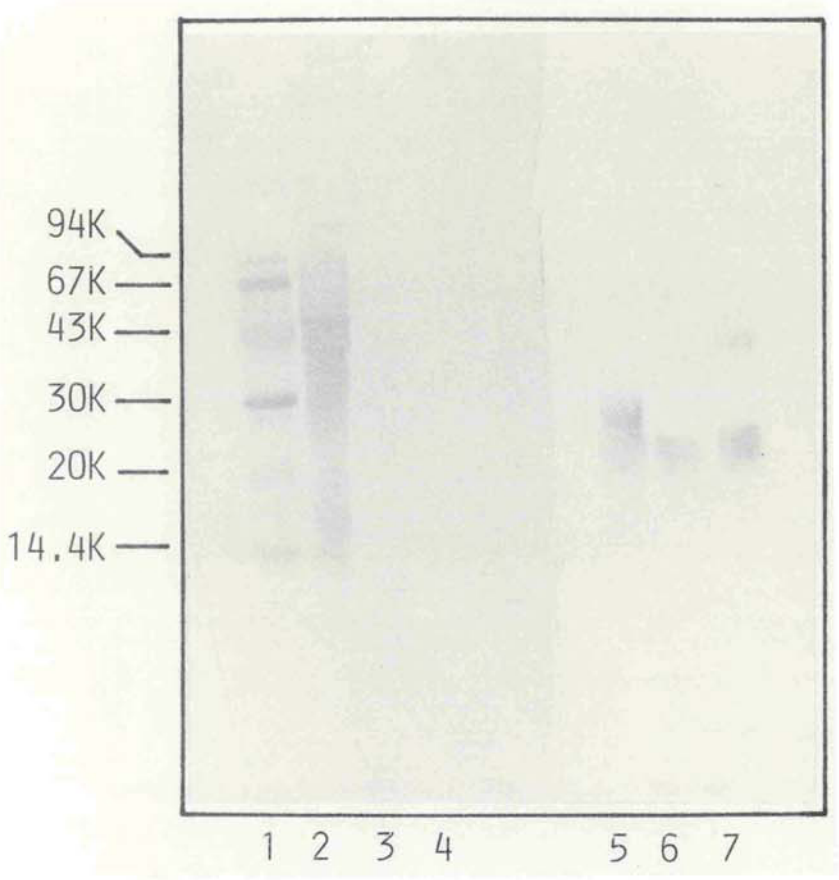
Lanes 5-7 were reacted with MCAb I4B1

Lane 1 Protein molecular weight markers (Pharmacia)

Lanes 2 and 5 10µg protein Inaba sonicated vibrios

Lanes 3 and 6 15ng of Inaba 35 A3 LPS

Lanes 4 and 7 5µg of Inaba 569 b LPS


94K
67K
43K
30K
20K
14.4K
1 2 3 4 5 6 7

4.9 V.cholerae proteins on SDS-PAGE

The Ogawa and the Inaba serotypes did not differ in the protein patterns obtained by the silver stain, the coomassie blue or the the amido black stains (Fig. 4.2b and c and 4.3 a). Some low molecular weight protein bands were dominant in the silver stained gel and the high molecular weight proteins did not stain very well. In the coomassie blue stained gel and the amido black stained blot there was an abundance of high molecular proteins and the low molecular weight proteins were barely detectable.

The silver stain used in this study (Section 2.3.5.5) stained both proteins and LPS. Glycoproteins have been reported to be most intensely stained by this method (Tsai and Frasch, 1982). The differential staining of the proteins by the three stains used may have been responsible for the different protein profiles.

4.10 Western blot of enzyme digests of LPS

The diffuse band of the V. cholerae LPS observed in the silver stained gels and the immunoblots raised the possibility of several related substances comigrating. Different glycosidases were used to study the effect on the migration of glycosidase-digests of LPS.

The V. cholerae LPS was digested with α-mannosidase, α-glucosidase and β-glucosidase as described in Section 2.3.5.8. The digested LPS was elecrophoresed and immunoblotted. Differences between the digested and the undigested molecules

were observed on blotting (Fig. 4.4a). α-mannosidase digest gave rise to two diffuse bands close to each other and in the same molecular weight range as that of the undigested molecule. In the β-glucosidase digest two bands were visible, one more dominant than the other. Digests from the α-glucosidase did not produce any change in the blotting pattern. Both Ogawa and Inaba LPS gave rise to similar bands on digestion with the glycosidases. Silver staining showed complex patterns as the enzyme preparations stained extensively themselves (Fig.4.4b). All the MCAbs reacted in a similar fashion.

4.11 Action of exoglycosidases on LPS

The exoglycosidases used in this study did not yield very conclusive results. The enzymes on their own showed multiple bands over a wide range of molecular weights with silver stain (Fig.4.4b). The presence of proteins affects the migration and staining of the LPS (Fig. 4.2) and hence it was difficult to interpret the results obtained in Fig. 4.4a.

Exoglycosidases would hydrolyse only the terminal sugar residues. This would lead to the shortening of the sugar chains on the core or the O-side chains. *V. cholerae* O-antigen is predominantly made up of (1→2) linked 4-amino-4,6-dideoxy-α-D mannopyranosyl residues (Redmond, 1979). The two diffused bands observed in the digest of α-mannosidase may have arisen from the partial digestion of the LPS molecule.

It was more difficult to interpret the results obtained from the digests of β-glucosidase. Glucose has been

Fig.4.4 a Western blots of enzyme digests of LPS

Inaba 35 A3 LPS digested with various enzymes (Section 2.3.5.8) was blotted and immunostained with protein A (Section 2.3.4.5.2).

Lane 1 Inaba LPS

lane 2 α-mannosidase digested Inaba LPS

Lane 3 α-mannosidase enzyme

Lane 4 β-glucosidase digested Inaba LPS

Lane 5 β-glucosidase enzyme

Lane 6 α-glucosidase (yeast) digested Inaba LPS

Lane 7 α-glucosidase (yeast) enzyme

Lane 8 α-glucosidase (rice) digested Inaba LPS

Lane 9 α-glucosidase (rice) enzyme

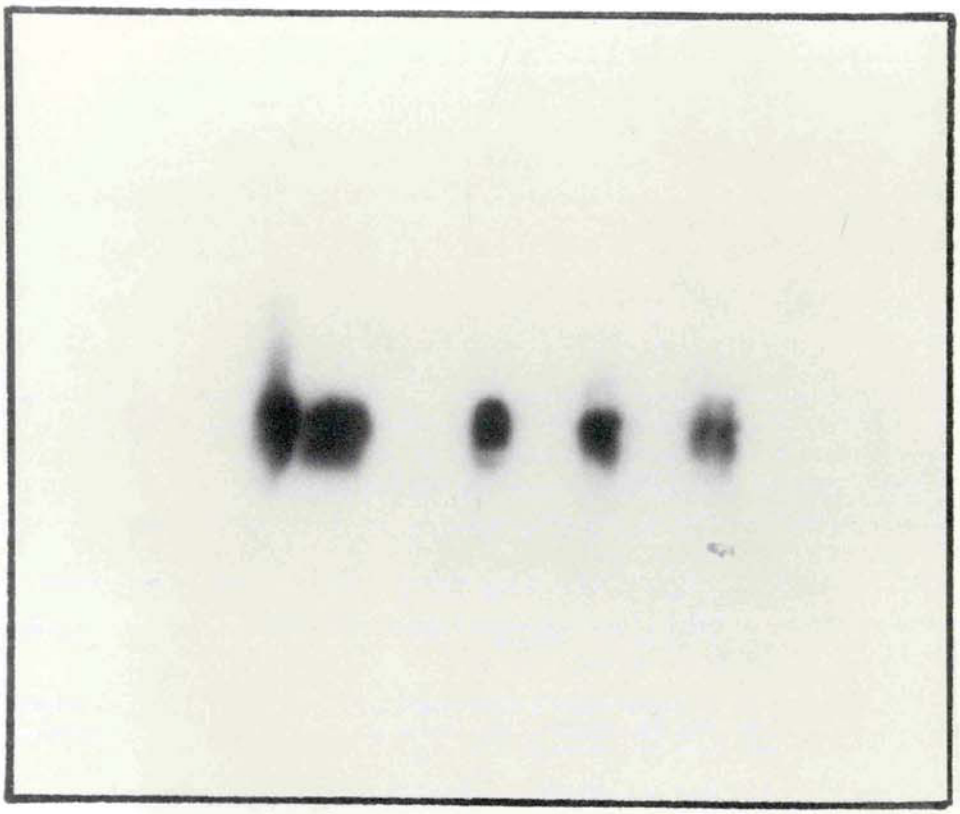

1 2 3 4 5 6 7 8 9

Fig.4.4 b Silver stain of enzyme digests of LPS

Enzyme digested LPS and the enzymes on their own were electrophoresed on 15% acrylamide gel, 10cm long.

Lane 1 α-glucosidase

Lane 2 β-glucosidase

Lane 3 Crude Inaba 35 A3 LPS

Lane 4 α-glucosidase digested Inaba 35 A3 LPS

Lane 5 β-glucosidase digested Inaba 35 A3 LPS


1
2
3
4
5

detected in the LPS of the V. cholerae (Raziuddin, 1979; Kenne et al, 1982), but details about its conformation are not available. The appearance of the two bands could be explained by partial hydrolysis of the LPS molecule, the substrate, β-glucoside being provided presumably by the core. This experimental approach is clearly severely hampered by two factors. The detailed structure of the LPS is not known and therefore the results are difficult to interpret. In addition, the enzymes employed were impure and any results obtained could be the result of the activity of unknown contaminants rather than the true enzyme. Consequently this line of investigation was not further pursued.

4.12 Analysis of LPS with lectins

Lectins are plant proteins which can bind to carbohydrates with a high degree of specificity. They are generally used to study the presence of sugar residues or the conformation of oligosaccharides in glycoproteins (Baenziger et al, 1979). Lectins have also been used in structural studies of gram-negative bacterial LPS (Kabir, 1982; Corbel et, al 1984). The lectins, Concanavalin A (ConA) and wheat germ agglutinin (WGA) were employed in this study.

4.12.1 Concanavalin A binding sites on V. cholerae LPS

V. cholerae LPS and the α- and β-glucosidase digests of the LPS were Western blotted and the blot was reacted with ^{125}I-Con A as described in Section 2.3.4.7.2. The film was

developed two days after exposure to the isotope. ^{125}I-Con A did not bind to the LPS or the enzyme digests of the LPS (Fig. 4.5). The radioiodinated con A did however bind to two bands of the enzyme β-glucosidase.

4.12.2 Wheat germ agglutinin binding studies on LPS

Ogawa and Inaba LPS were electrophoresed on a 15% polyacrylamide gel along with some molecular weight protein markers. The gel was overlaid with ^{125}I-WGA as described in section 2.3.4.7.1. The film was developed 20 days after exposure to the radioisotope. The iodinated WGA bound to the glycoprotein, ovalbumin and also to phosphrylase b among the protein markers but not to the LPS (Fig. 4.6). Phosphorylase b is not a glycoprotein but the product obtained from Pharmacia kit of protein markers may have some glucan residues as suggested by Dubray and Bezard (1982).

4.13 Lectin-LPS interaction

The work undertaken here with the lectins demonstrated that neither Con A nor WGA were capable of binding to vibrio LPS. Kabir (1982) had reported the precipitation of alkali-treated Ogawa LPS with Con A and N-acytelated LPS with WGA in capillary tubes. Two of the methods used in this study were, the treatment of a blot and a gel with lectins. To obtain a minimal amount of background the gel or the blot had to be washed several times with wash buffer containing Tween 20 (Section2.3.4.7.1).

If the binding of the lectins to the bacterial LPS was of

Fig. 4.5 Concanavalin A binding sites on V.cholerae LPS

LPS and enzyme digests of LPS were electrophoresed and blotted. The blot was treated with radioiodinated con A (Section 2.3.4.7.2)

Lane 1 Ogawa LPS

Lane 2 α-glucosidase digested Ogawa LPS

Lane 3 β-glucosidase digested Ogawa LPS

Lane 4 α-glucosidase enzyme

Lane 5 β-glucosidase enzyme

Lane 6 Inaba LPS

Lane 7 α-glucosidase digested Inaba LPS

Lane 8 β-glucosidase digested Inaba LPS


1 2 3 4 5 6 7 8

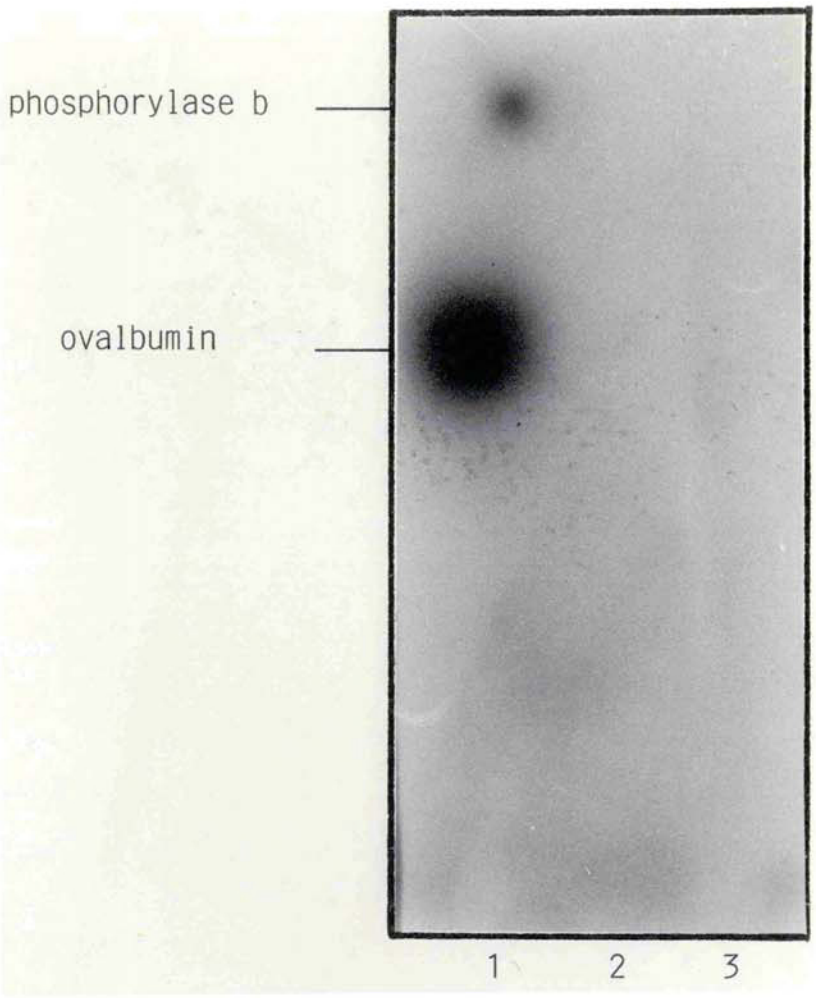
Fig. 4.6 Wheat germ agglutinin binding sites on V.cholerae LPS

LPS from Ogawa and Inaba were electrophoresed and treated with radioiodinated WGA.

Lane 1 Standard protein molecular weight markers

Lane 2 Ogawa LPS

Lane 3 Inaba LPS


phosphorylase b
ovalbumin
1
2
3

low affinity the possibility of them being washed from the gel or the blot was high.

The observations from this work did not indicate any binding of the lectins to the LPS. The glucosamines residues detected by Kabir (1982) were the residues of the lipid A which the author had exposed by pretreating the LPS with alkali.

4.14 Resolution of V. cholerae LPS

In contrast to LPS preparations from other gram negative bacteria, resolution of the O-antigen side chains of V. cholerae LPS was difficult to obtain under normal conditions of gel electrophoresis. The migration of the complex O-side chain of the V. cholerae LPS gave a diffuse, but definitely not single band (Section 4.4, Fig. 4.2). It was clearly more homogenous than other gram-negative bacterial LPS molecules analysed on SDS-polyacrylamide gels (Hitchcock and Brown, 1983, Schiller et al, 1984). However, the broad and diffuse band of the O-antigen suggested the existence of heterogeneity at a finer level. Resolution of the microheterogeneity in the LPS molecule was undertaken by making some modifications in the conditions of SDS-PAGE.

4.14.1 Resolution of LPS at high percentage of acrylamide and a long electrophoresis time

E.coli (serotype 0127 B8) LPS (Sigma) and crude V. cholerae LPS were electrophoresed on a 20% polyacrylamide gel

of 20 cm length as described in Section 2.3.5.6.1. The gel was then stained for LPS.

E.coli (0127 B8) LPS resolved into numerous bands in the classical "ladder". From the lower end of the gel the different components of the LPS were the lipid A, the core and then the O-side chains with increasing molecular weight as the number of repeating units attached to it increased (Fig. 4.7). The V. cholerae LPS had again not been resolved with respect to the O-side chain. It exhibited the lipid A and the core as a doublet which had the same mobility as the lipid A of E. coli. On careful examination of the stained O-region of the LPS it was possible to detect a few bands at the trailing end.

It became apparent, after a large number of experiments, that it was not the use of a high percentage gel, but rather the long electrophoresis which allowed the LPS to be further resolved.

4.14.2 Resolution of LPS on a 15% gel with a long electrophoresis time

V. cholerae LPS was electrophoresed on a 15% acrylamide gel as described in Section 2.3.5.6.2 to improve upon the resolution achieved so far. The gel was then silver stained as before. This resolved the V. cholerae O-side chains and revealed the existence of at least 8-9 discrete bands (Fig. 4.8). The lipid A component was allowed to run off the end of the gel. The separation of the complex O-side chains was concentration dependent. At low concentrations the separated bands did not

Fig. 4.7 Silver stain of LPS on a 20cm long 20% SDS-polyacrylamide gel

E. coli (0127 B8), Ogawa NIH 41 and Inaba 35 A3 LPS were electrophoresed as described in Section 2.3.5.6.1.

Lane 1 10µg *E. coli* LPS

Lane 2 7.5µg *E. coli* LPS

Lane 3 5µg *E. coli* LPS

Lane 4 5ng Ogawa LPS

Lane 5 3.7ng Ogawa LPS

Lane 6 2.5ng Ogawa LPS

Lane 7 10ng Inaba LPS

Lane 8 7.5ng Inaba LPS

Lane 9 5ng Inaba LPS

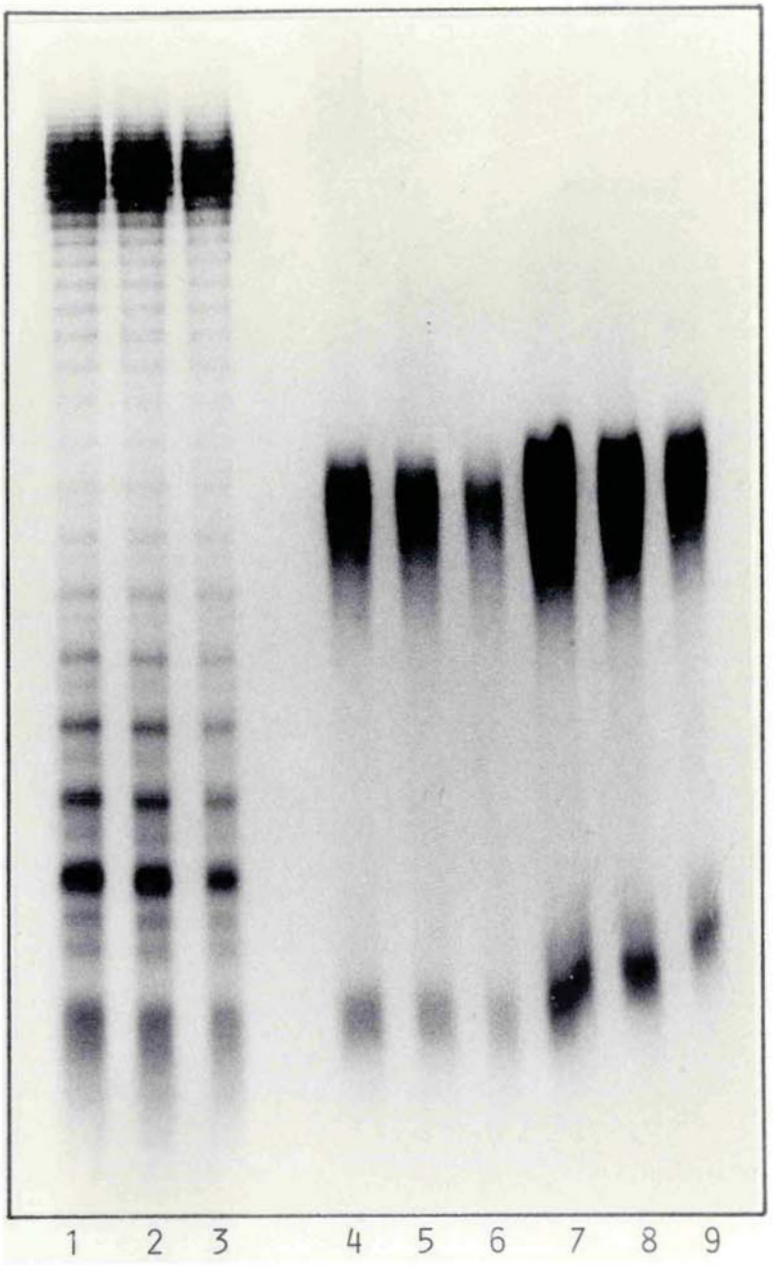

1 2 3 4 5 6 7 8 9

Fig. 4.8 Silver stain of LPS on a 20cm long 15% SDS-polyacrylamide gel

E. coli 0127 B8, Ogawa NIH 41 and Inaba 35 A3 LPS were electrophoresed on 15% gel as described in Section 2.3.5.6.2.

Lane 1 10µg *E. coli* LPS

Lane 2 7.5µg *E. coli* LPS

Lane 3 5µg *E. coli* LPS

Lane 4 5ng Ogawa LPS

Lane 5 3.7ng Ogawa LPS

Lane 6 2.5ng Ogawa LPS

Lane 7 10ng Inaba LPS

Lane 8 7.5ng Inaba LPS

Lane 9 5ng Inaba LPS


1 2 3 4 5 6 7 8 9

stain well (Fig. 4.8, lane 6) and at high concentrations the slow moving bands were overstained and hence remained indistinguishable (Fig. 4.8, lane 7). The best separation and staining of the LPS was obtained at concentrations of 10-15 ng (Fig. 4.8, lanes 8 and 9).

The E. coli LPS was less suited for this system as the sharp bands obtained in the 20 % acrylamide gel (Section 4.14.1) were diffuse, though each diffuse band showed little apparent microheterogeneity.

4.14.3 Electrophoresis of LPS on a DNA sequencing gel

On a gel system like that described in section 4.14.2, the O-side chains of the V. cholerae LPS were resolved by allowing the high mobility band of lipid A to run off the gel. To achieve a good resolution and retain the lipid A band in the gel, a DNA sequencing gel of 42 cm length was employed. On a long gel greater amounts of LPS were needed for the optimum separation and staining of the LPS. The electrophoresis was carried out as described in Section 2.3.5.6.3. The gel was cut in two halves before being stained for LPS. The upper half of the gel did not contain any stained material and the lower half had both the lipid A and the O-region (Fig. 4.9). Under these conditions of separation at least ten distinct bands were visible in the O-region. It was apparent that at low concentrations of LPS the high mobility bands of the O-side chain were not detectable.

The lipid A part of the molecule was also resolved into three bands not observed before. The middle band of the

Fig. 4.9 Silver stain of V.cholerae LPS

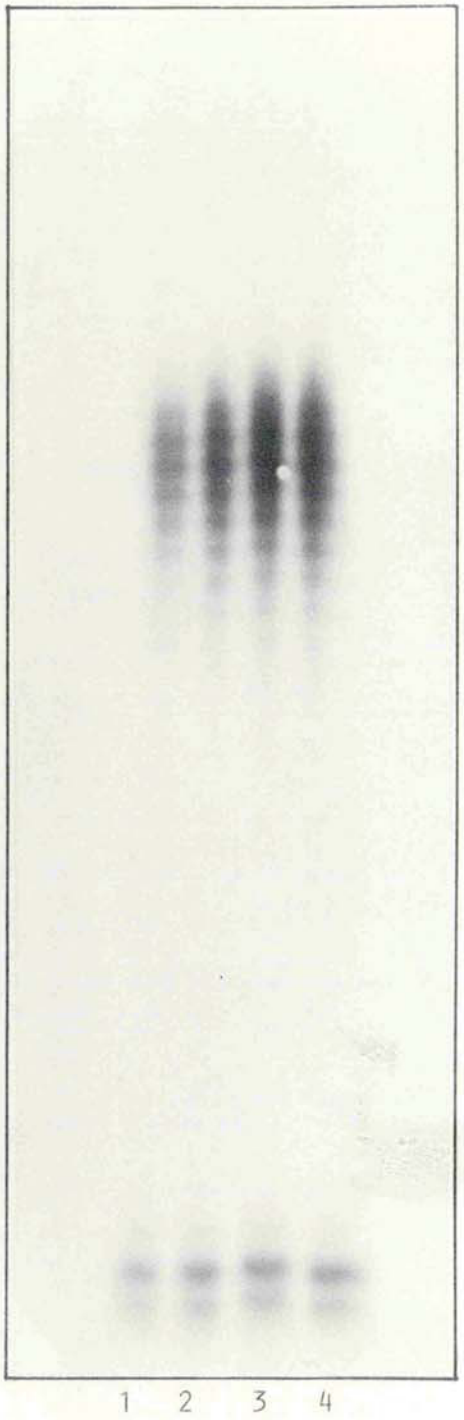
Crude Inaba 35 A3 LPS was electrophoresed on a 15% SDS-polyacrylamide gel of length 42cm as described in Section 2.3.5.6.3. The top 20cm of the gel which contained no material reacting with the stain have not been included in the photograph.

Lane 1 15ng Inaba LPS

Lane 2 20ng Inaba LPS

Lane 3 22.5ng Inaba LPS

Lane 4 25ng Inaba LPS


1 2 3 4

three was most intensely stained followed by the lower most band and then the top most band.

4.15 Immunoblots of resolved LPS

4.15.1 Enzyme detection of immunoblots

Immunoblots of gram-negative bacterial LPS molecules have been reported by various workers (Sidberry *et al*, 1985; Poxton *et al*, 1985; Sturm *et al*, 1984). The antibodies essentially recognise all the polymeric bands of the O-antigen. The MCAbs raised against *V. cholerae* were reacted with the resolved LPS to analyse their reactivity against the various O-antigen bands. The crude LPS from either serotype of *V. cholerae* were resolved on 15% acrylamide gels as described in Section 2.3.5.6.2 and blotted. The blots were reacted with MCAbs and developed by the ELISA technique using 4-chloronaphthol as insoluble substrate (Section 2.3.4.5.1).

A good immunoblot of the LPS was difficult to achieve because of the poor efficiency of blotting of the resolved O-antigens. The sites of the O-antigen recognised by the MCAbs are seen in Fig. 4.10. All the samples were electrophoresed and blotted together. In Fig.4.10b, the stripes of the O-antigen can just be detected. In the rest of the blots the LPS appeared as a long streak with some portions of it more reactive than others. There clearly seemed to be two to three regions in the area of the O-antigen which bound more antibody. The smearing of the O-antigen observed in Fig.4.10a, lane 3 was not uncommon. The enzyme linked detection system is one of limited sensitivity,

Fig. 4.10 a Immunoblot of V.cholerae LPS

Ogawa NIH 41 and Inaba 35 A3 LPS were electrophoresed (Section 2.3.5.6.2) and blotted. The blots were reacted with six MCAbs and stained with 4-chloronaphthol (Section 2.3.4.5.1).

Lanes 1,3,5,7,9 and 11 15ng Ogawa LPS

Lanes 2,4,6,8,10 and 12 15ng Inaba LPS

Lanes 1 and 2 reacted with I1A1

Lanes 3 and 4 reacted with I4B1

Lanes 5 and 6 reacted with I4C3

Lanes 7 and 8 reacted with O4A6

Lanes 9 and 10 reacted with O3D1

Lanes 11 and 12 reacted with O4D2

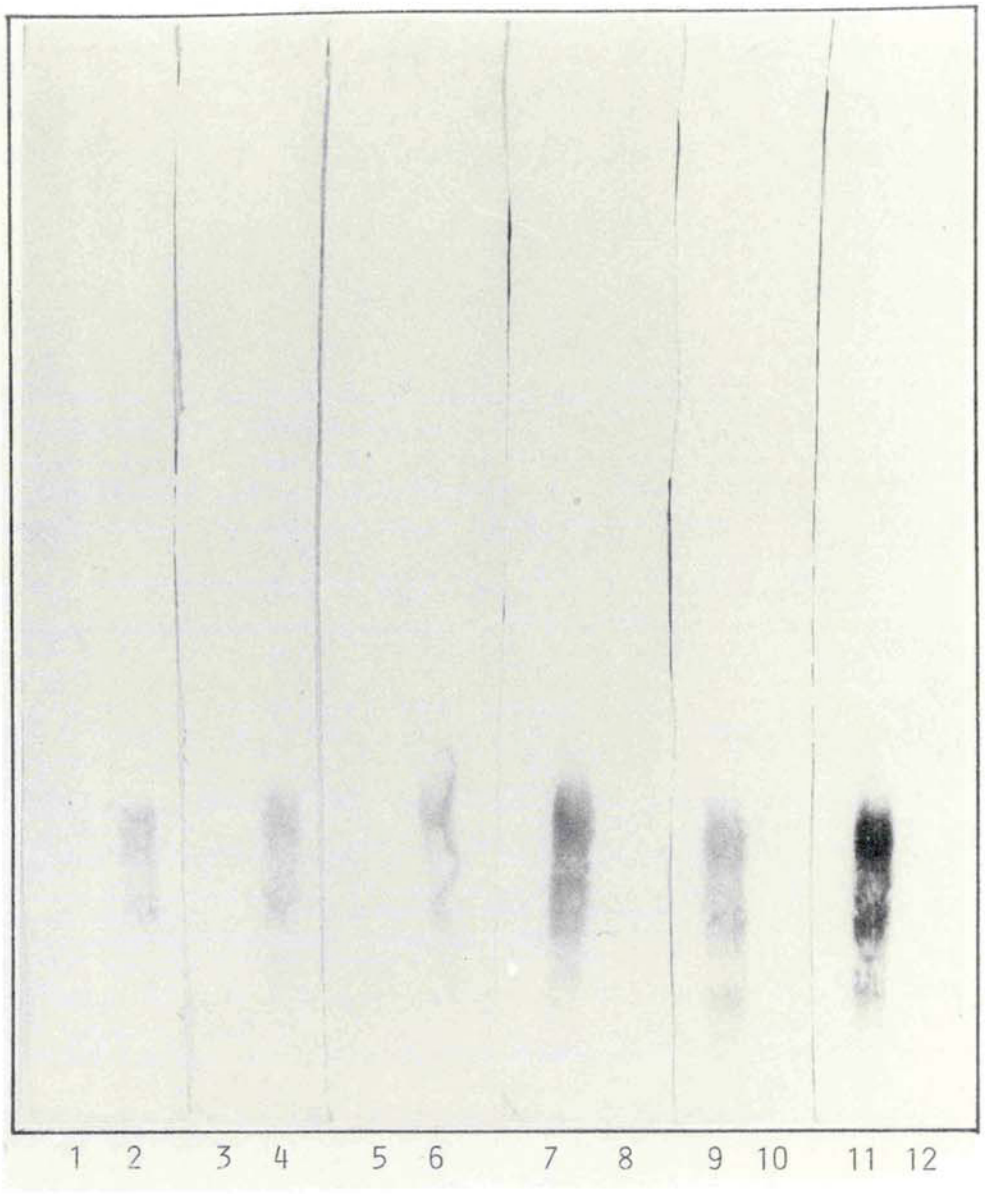

1 2 3 4 5 6 7 8 9 10 11 12

Fig. 4.10 b) Immunoblot of V.cholerae LPS

Magnified view of stained portion of Lane 2 of Fig.4.10 a).

although good resolution and a higher sensitivity technique would be more suitable for the analysis of LPS which only resolves at low concentration.

4.15.2 Immunoblots using ^{125}I-Protein A

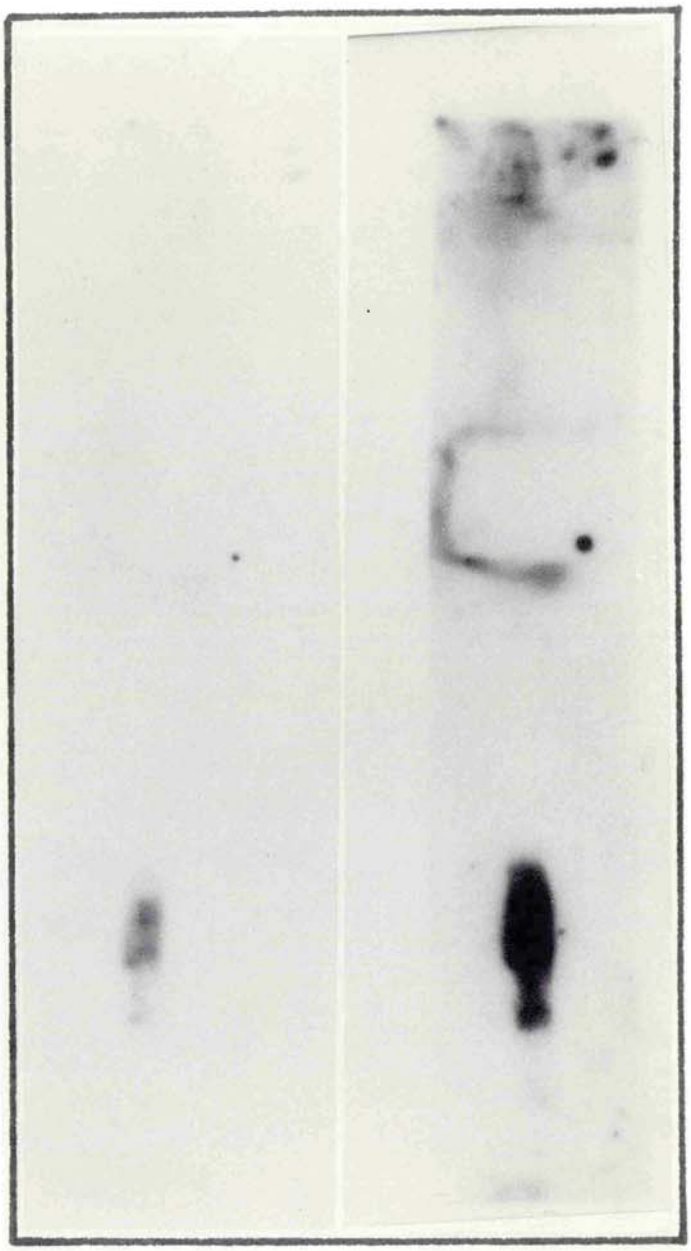
The method employed here involved the use of a double sandwich system (Section 2.3.4.5.2) by using rabbit anti-rat specific antiserum followed by iodinated protein A after the blot was treated with the MCAbs. This method was employed in order to attempt to improve upon the immunoblots obtained by the enzyme detection technique. Various concentrations of the LPS were used. Low amounts resulted in faint streaks with no apparent signs of the ladder like appearence of the antigen. High amounts also did not show the stripes observed in a silver stained gel. In Fig. 4.11 a and b, Inaba LPS was resolved on a gel (Section 2.3.5.6.2) and transferred onto a nitrocellulose paper. The blot was reacted with MCAb I1A1 and detected using iodinated protein A. Films were exposed to the blot for different time intervals. A study of the blot from films developed after various exposure times indicated regions of preferences on the antigen for antibody binding. Two or three "hot spot" regions with inert regions between them are apparent, confirming the results obtained by enzyme blots (Fig.4.10). The sensitivity of this detection system was much greater and it could be used with smaller amounts of antigen. However, the resolution was no finer, presumably due to the path length of the gamma rays from the ^{125}I isotope being too great and leading to blurring effects..

Fig. 4.11 Immunoblot of V.cholerae LPS

Inaba 35 A3 LPS was electrophoresed (Section 2.3.5.6.2) and blotted. The blot was treated with MCAb I4C3 and developed with radioiodinated protein A (Section 2.3.4.5.2).

a) Lane 1 Inaba LPS reacted with I4C3 and developed after 3 days of exposure to the isotope.

b) Lane 1 Inaba LPS reacted with I4C3 and developed after 7 days of exposure to the isotope.



(a) 1 (b) 1

4.16 Serotype Specificity of MCAbs

The MCAbs raised in this study to *V. cholerae* did not react with *B. abortus* or *E. coli* (strain ML 308) on ELISA (Section 3.6). The antibodies were tested for cross-reactivity on blots of sonicated *B. abortus* organisms, *E. coli* (0111 B4) LPS and *S. typhimurium* LPS (Fig. 4.12). None of the antibodies showed any cross reactivity with this diverse panel of antigens on blotting.

4.17 Relative mobilities of LPS molecules on SDS-PAGE

Ogawa and Inaba LPS gave identical mobility with respect to lipid A, core and the O-antigen on SDS-PAGE. *S. typhimurium* and *E. coli* (0111 B4) LPS have been very well characterised. The mobilities of these two LPS molecules were therefore compared with that of Ogawa and Inaba LPS (Fig. 4.13). The whole range of the *V. cholerae* LPS was within the space of five repeat units of *S. typhimurium*.

4.18 Analysis of hydrolysed LPS on SDS-PAGE

The lipid A of LPS is linked to the terminal sugar of the core by a ketosidic bond which is acid labile. Most gram-negative bacterial LPS molecules (Jann *et al*, 1975) including *V. cholerae* LPS (Kenne *et al*, 1982) have been dissected by a preliminary acid hydrolysis. Crude Ogawa and Inaba LPS were hydrolysed as described in Section 2.3.5.9. The hydrolysed product was extracted with ether twice. The ether soluble product

Fig. 4.12 Serotype specificity of MCAbs as exhibited on Immunoblots

Sonicated bacteria or LPS were electrophoresed (Section 2.3.5.6.2) and electroblotted. The blot was immunostained as described in Section 2.3.4.5.1.

Lanes 1-4 reacted with MCAb O3D1

Lanes 5-9 reacted with MCAb I4B1

Lanes 1 and 5 10µg protein of sonicated *B. abortus*

Lanes 2 and 7 10µg *E. coli* LPS

Lanes 3 and 4 15 and 10ng Ogawa LPS

Lanes 6 and 8 7 and 15ng Inaba LPS

Lane 9 10µg *S. typhymurium* LPS

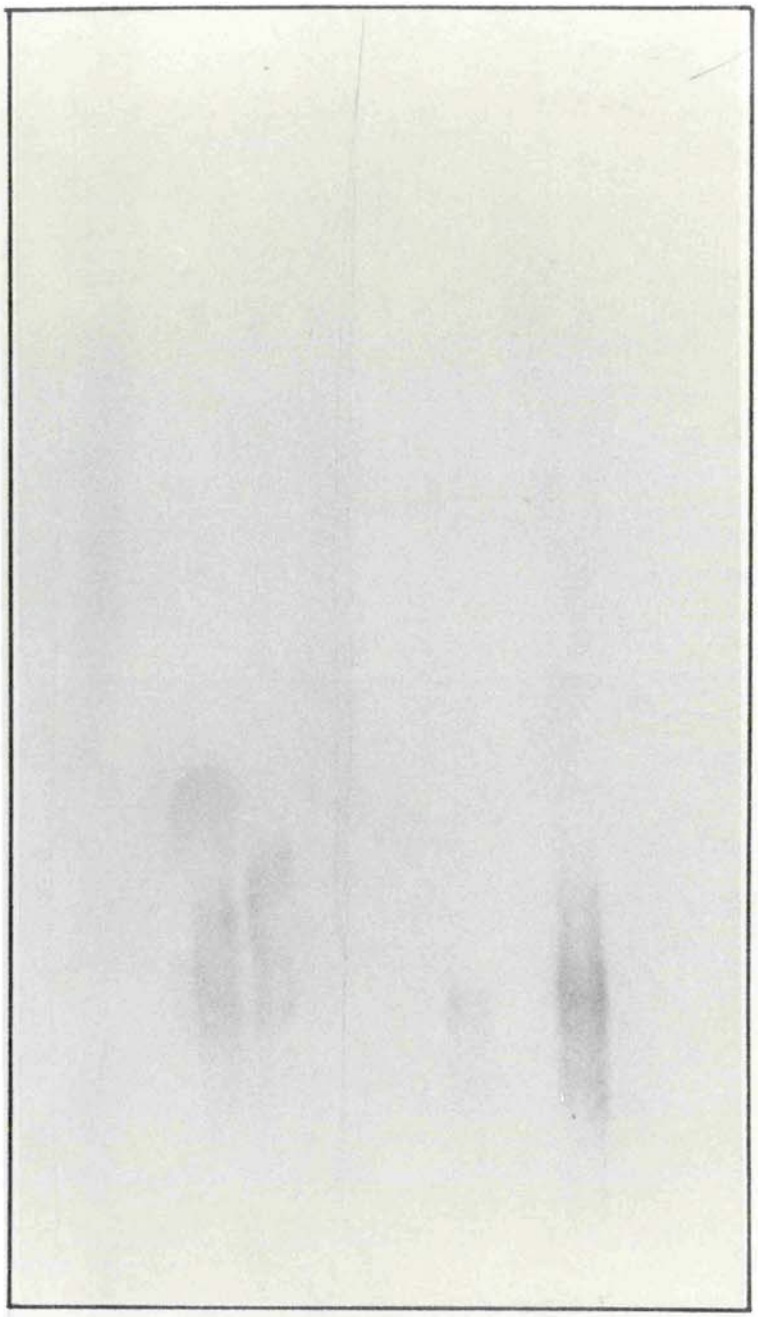

1 2 3 4 5 6 7 8 9

Fig. 4.13 Silver stain of LPS

LPS from various bacteria were electrophoresed and silver stained.

Lane 1 5ng Ogawa LPS

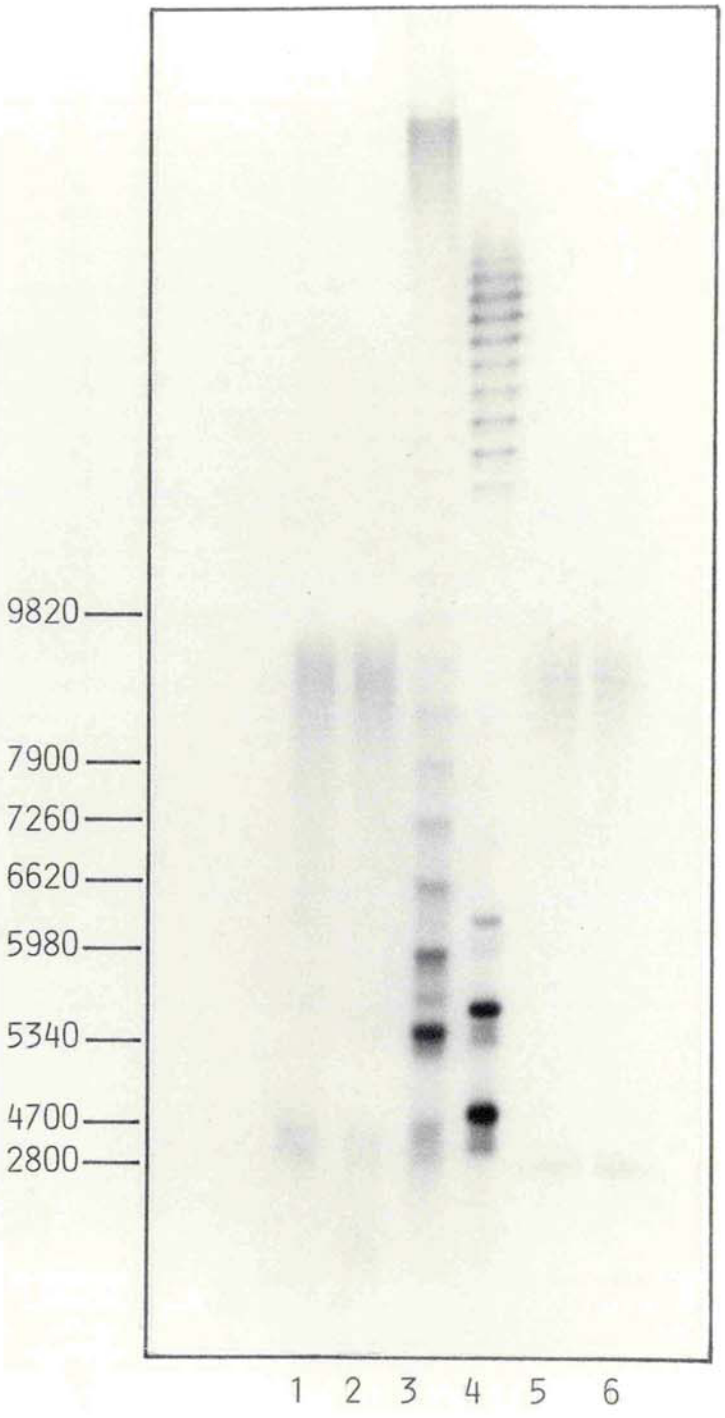
Lane 2 5ng Inaba LPS

Lane 3 10µg *S. typhimurium* LPS

Lane 4 10µg *E. coli* LPS

Lane 5 Acetic acid hydrolysed Ogawa LPS

Lane 6 Ether extracted aqueous phase of hydrolysed Ogawa LPS


9820
7900
7260
6620
5980
5340
4700
2800
1
2
3
4
5
6

and the aqueous phase were electrophoresed. The ether phase obtained did not stain with silver nitrate (not shown). The aqueous phase before and after ether extraction was stained and showed no difference between serotype. However, the doublet of the lipid A-core region was reduced to a single band in both cases (Fig.4.13). The Inaba LPS, not shown in the Figure, gave the same results. While the hydrolysed sample appeared in this case to be resolved with greater clarity, further experiments showed this to be due to the concentration effect (Section 4.14.2) with the hydrolysed material being present in slightly lower amount.

4.19 Features of V. cholerae LPS

The resolution of the LPS obtained in this study demonstrates that like other Gram-negative bacterial LPS molecules, V. cholerae LPS is also heterogenous, though the extent of heterogeneity observed is much smaller. Unlike E. coli and S. typhimurium LPS, V. cholerae LPS has a restricted preferred range of repeating units, starting well above the one repeat unit shown by these molecules.

The V. cholerae O-antigen is known to be composed of perosaminyl residues, the amino groups of which are acylated by 3-deoxy-L-glycero-tetronic acid (Kenne et al, 1982). The repeat unit, however has not been identified to date. The close similarities observed between the O-chain bands on silver stained gels (Fig. 4.8 and 4.9) suggested very small differences in molecular weight between them. Such differences on the DNA

sequencing apparatus correspond to an increment of one base in the DNA molecule. By analogy, the difference between LPS bands was in the region of a single sugar residue.

The dependence on concentration of the resolution of the LPS (Section 4.14.2) confirmed that the differences between the bands were very small. At low concentrations, resolution was good but only a small number of bands were visible. At higher concentrations, these original bands became blurred but further resolved bands could be identified below the original (Fig. 4.9). Consequently, it is difficult to place an estimate on the total number of bands present in any sample. If the increment between bands is taken as a single repeating unit, this suggests that, while a large number of repeating units is the favoured structure for most of the LPS molecules, there are also molecules present with a lower and possibly much lower, number of repeating units.

The immunoblots of the LPS (Fig. 4.10 and 4.11) illustrate a different pattern from that obtained in the silver stained gels. Certain regions of the O-antigen in the immunoblots bound more antibody than others. This might suggest that some bands are more antigenic than others. However, it is also possible that this result is essentially an experimental artefact due to some of the bands being less efficiently blotted on to the nitrocellulose paper.

The mobility of the lipid A-core complex of the V. cholerae LPS was very similar to that of the S. typhimurium and the E. coli LPS (Section 4.14.1).

The disappearance of one band from the doublet of the lipid A-core of V. cholerae LPS on acid hydrolysis (Section 4.18) strengthens the tentative identification of the high mobility doublet as that of the lipid A-core complex. However, the reduction of the doublet to a single band was observed even before the ether extraction of the hydrolysed LPS. This could mean that the unbound lipid A was present in very small amounts after hydrolysis and was consequently not detected.

ANALYSIS OF THE SPECIFICITIES OF THE MONOCLONAL ANTIBODIES

The anti-*V. cholerae* MCAbs were shown to react with the side-chains of the O-antigen of the respective serotypes only, in the Western blots (Section 4.8). The specificity of the MCAbs was further analysed under various assay systems to yield more information about the LPS as antigen and the antigen-antibody binding.

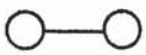
5.1 Competition assay (ELISA) with vibrios and crude LPS

Crude LPS and vibrios were used as competing antigens in an attempt to block the binding sites of the antibodies to the whole vibrios coated on ELISA plates. Competing antigen was incubated with the MCAbs (Section 2.3.3.2) and the mixture was added to the ELISA plates coated with either Ogawa or Inaba vibrios. The antibodies not bound to any of the competing antigens were free to react with the vibrios.

Fig. 5.1a illustrated that homologous serotype vibrios or LPS though from a different strain inhibited I4B1 from binding to the vibrios coated on ELISA plates. Fig.5.1 b and c demonstrated that the binding of the MCAbs, O4A6, O3D1 and O4D2 to the Ogawa

Fig.5.1 a <u>Competition ELISA with vibrios and purified LPS</u>

Inhibition of binding of I4B1 and O3D1 MCAbs to Inaba 35 A3 and Ogawa NIH 41 vibrios respectively were studied with homologous vibrios and Inaba 569 B LPS (Sigma). Each value plotted in the graph is a mean of three assays.


I4B1 competed with Inaba 35 A3 vibrios


I4B1 competed with Inaba 569 B LPS


O3D1 competed with Ogawa NIH 41 vibrios


O3D1 competed with Inaba 569 B LPS

Background reaction was below 0.1 O.D.

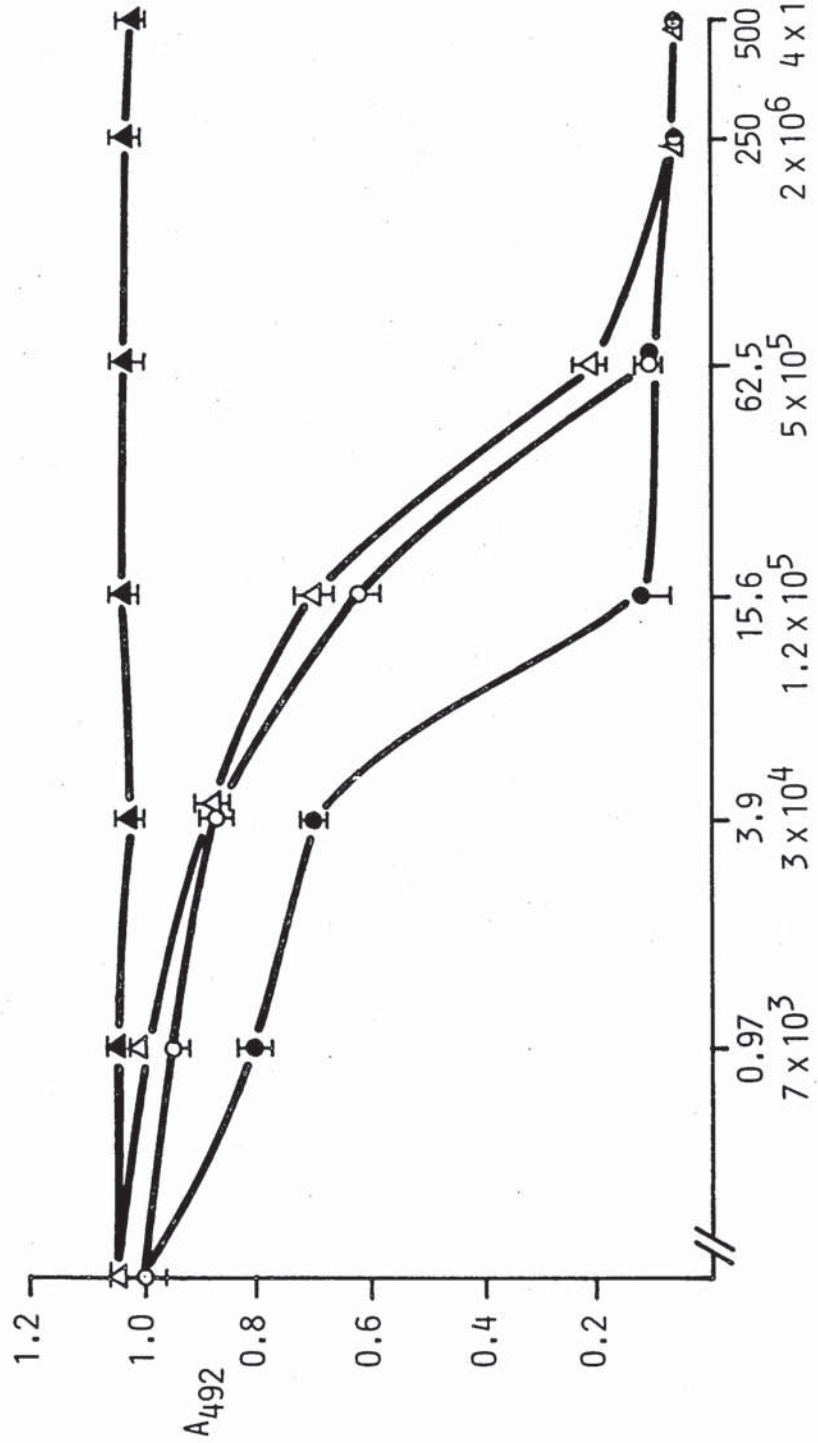
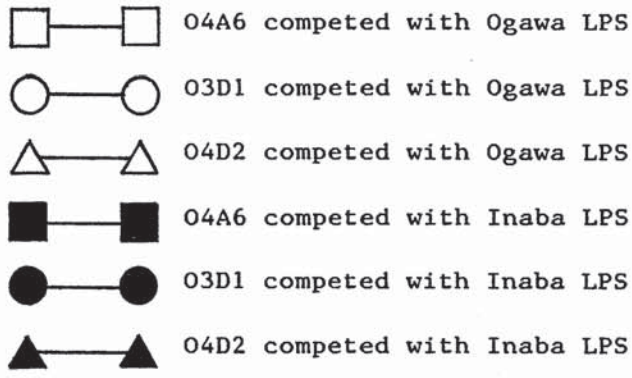

1.2
1.0
0.8
0.6
0.4
0.2
A_{492}
0.97
7×10^3
3.9
3×10^4
15.6
1.2×10^5
62.5
5×10^5
250
2×10^6
500 ng of LPS
4×10^6 no. of vibrios

Fig.5.1 b Competition ELISA with crude V. cholerae LPS

Crude V. cholerae LPS from Ogawa NIH 41 and Inaba 35 A3 were used as inhibitors as described in Section 2.3.3.2. Each value represents the mean of three assays.



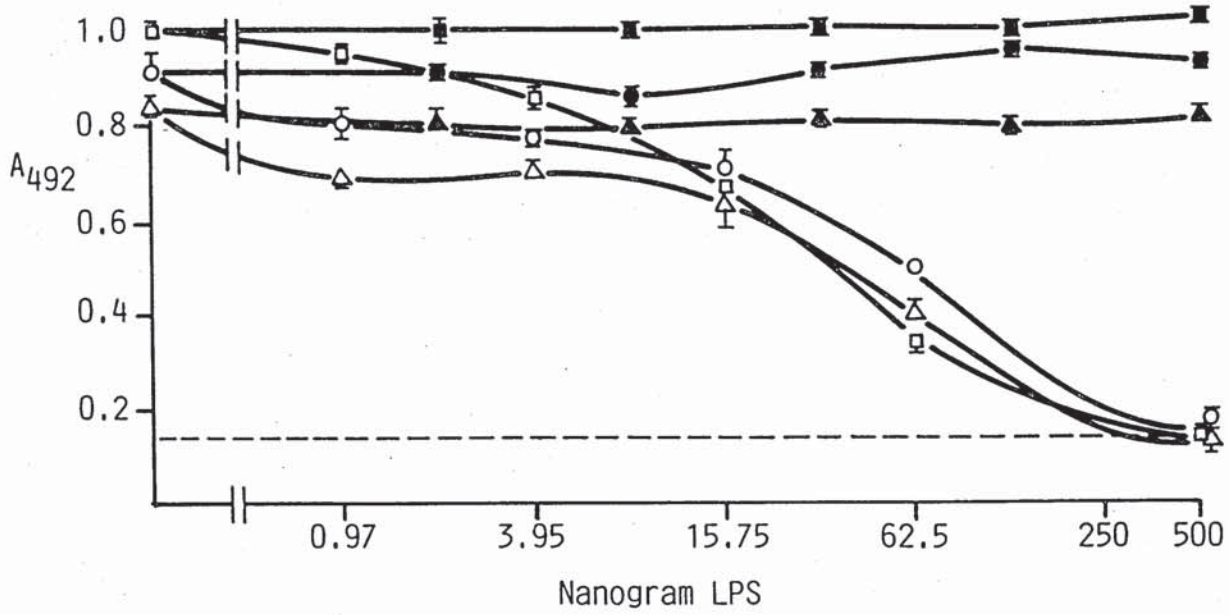
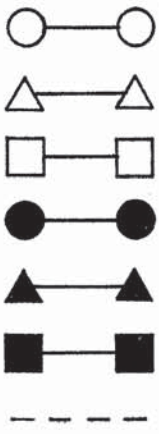

1.0
0.8
0.6
0.4
0.2
A_{492}
0.97
3.95
15.75
62.5
250
500
Nanogram LPS

Fig. 5.1 c Competition ELISA with crude V. cholerae LPS

Crude V. cholerae LPS from Ogawa NIH 41 and Inaba 35 A3 were used as inhibitors as described in Section 2.3.3.2. Each value plotted represents the mean obtained three assays.



I1A1 competed with Ogawa LPS

I4B1 competed with Ogawa LPS

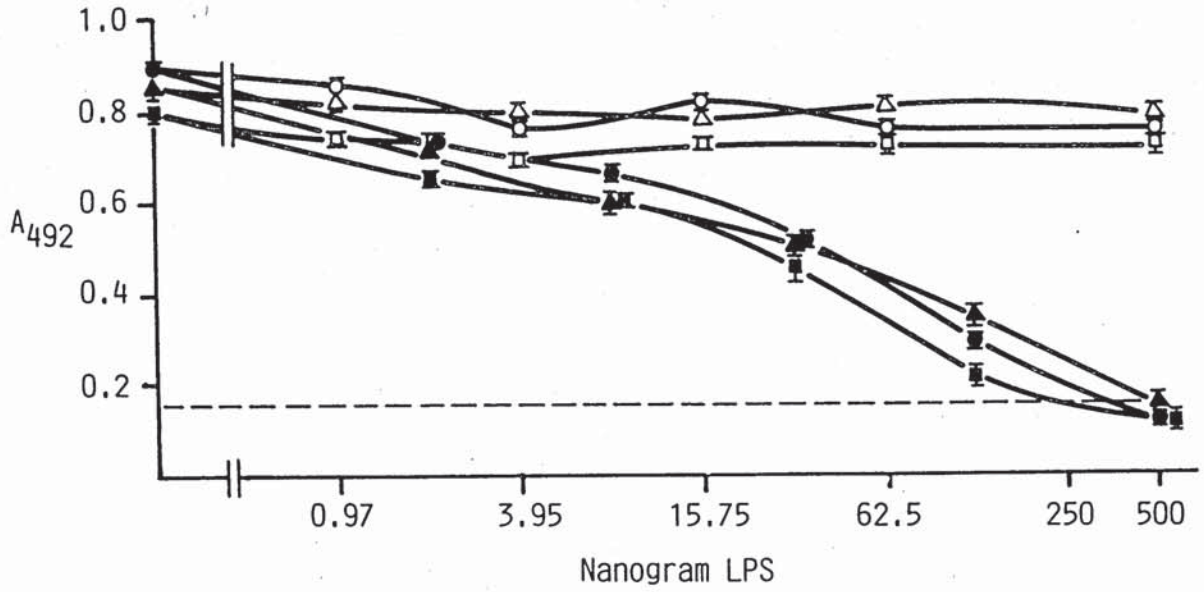
I4C3 competed with Ogawa LPS

I1A1 competed with Inaba LPS

I4B1 competed with Inaba LPS

I4C3 competed with Inaba LPS

Background reaction


1.0
0.8
0.6
0.4
0.2
A_{492}
0.97
3.95
15.75
62.5
250
500
Nanogram LPS

vibrios and the I1A1, I4B1 and I4C3 to the Inaba vibrios could be completely blocked with the homologous LPS. On the other hand even high quantities of heterologous LPS did not inhibit the binding of either the Ogawa or the Inaba series of MCAbs to their specific serotypes. The similarity of Inaba 569B LPS and Inaba 35 A3 LPS was illustrated by the fact that both were capable of inhibiting the Inaba positive MCAbs from binding to the Inaba 35 A3 vibrios.

5.2 Analysis of MCAb specificity by indirect immunofluorescence (IF) assay

IF assays were similar to ELISA assays with two exceptions. First, the organisms were fixed on to the slides with alcohol, second Tween 20 was not included in the wash buffer (Section 2.3.3.3).

MCAbs O4A6, O3D1 and O4D2 reacted very strongly with the Ogawa organisms (Fig. 5.2) and did not react at all with the Inaba organisms (Table 5.1). MCAbs I1A1, I4B1 and I4C3 exhibited a different trend in their reactivities towards the vibrios. Each one of them reacted strongly with the Inaba vibrios but also reacted with the Ogawa organisms with about half the homologous signal strength (Table 5.1).

The cross-reaction of the three Inaba positive MCAbs in the IF assay contrasted with the high degree of specificity which they exhibited in the ELISA and the Western blots (Section 3.6 and 4.8).

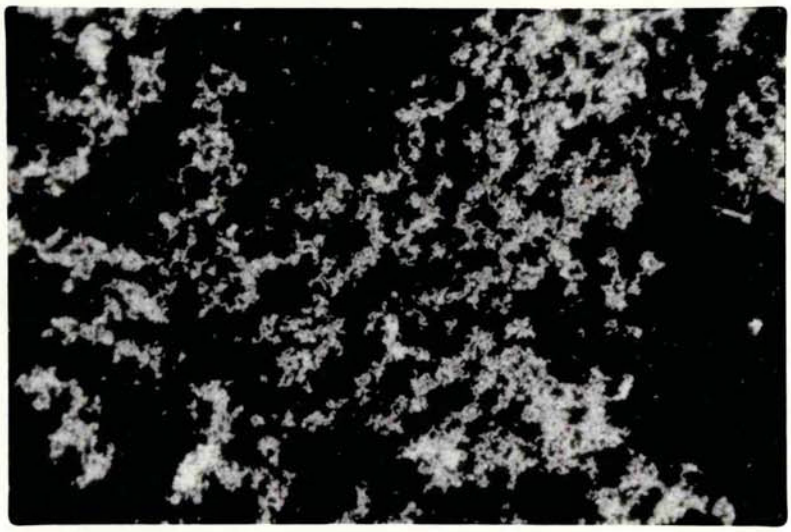


Fig. 5.2 Ogawa vibrios detected by immunofluorescent staining

Ogawa vibrios were reacted with MCAb O4A6 followed by anti-rat IgG fluorescein conjugate.

Table 5.1 Quantitation of indirect immunofluorescence assay with MCAbs

Quantitation of immunofluorescence signal was carried out on a LKB photometer (Section 2.3.3.3). The background was obtained from a mean of ten separate readings. The fluorescence obtained by the MCAb reaction was a mean of 25 readings. Background values were deducted from each set of reading for obtaining the mean values.

Monoclonal Antibody	Quantitation of IF	
	Ogawa	Inaba
Background	1.87	1.87
O4A6	38.00 ± 17	0.16 ± 0.4
O4D2	37.00 ± 17	0.15 ± 0.16
O3D1	35.00 ± 18	0.14 ± 0.1
I1A1	6.00 ± 4	14.00 ± 3.6
I4B1	5.60 ± 2.5	14.00 ± 3.4
I4C3	4.5 ± 1.3	9.70 ± 2.4

5.3 ELISA with varying washing procedures

The fact that I1A1, I4B1 and I4C3 cross-reacted significantly with the Ogawa vibrios in the indirect IF assay while they were apparently totally serotype specific on ELISA, competition ELISA and immunoblotting, was of considerable interest. While there are several differences in the protocols such as the labelled second antibody, the nature of the solid support, and the detection system, two factors seemed the most relevant and simple to test. The first was fixation. Fixation of antigens with formaldehyde is known to cause covalent chemical changes in the epitopes which can lead to alterations in specificity profile (Milstein et al, 1983) and this could potentially have been the reason for the cross-reaction observed. However, only alcohol, a mild fixative reagent was used to fix the vibrios and the much more drastic chemical procedures involved in LPS purification and immunoblotting had yielded an antigen which reacted with absolute serotype specificity. To test the second major possibility, that differential washing was responsible for the discrepancy in cross-reaction with assay, the ELISA wash buffer was modified to contain no Tween-20. The normal ELISA wash buffer (Section 2.3.3.1) was also used and the the number of times a plate was washed after each incubation was varied. If the Inaba series of antibodies were capable of cross reaction with lower affinity sites on Ogawa, it seemed likely that this might be detected by differential washing procedures, particularly in the presence or absence of the detergent.

The assay was carried with the MCAb I4C3. The different washes included, two washes with only PBS, two washes with PBS-Tween and six washes with PBS-Tween. The increase or decrease in the binding of the I4C3 antibody to Ogawa or Inaba under the modified protocols were primarily due to the changes in the background values. The background value steadily decreased as the washing procedure was made more stringent. The PBS wash had a background value of 0.2 which decreased to a value of 0.06 with six washes of PBS-Tween. The ratio of the Ogawa to Inaba binding did not change significantly with the alterations in the wash protocols and Tween-20 had no significant effect on relative affinity (Table 5.2; Fig 5.3). Consequently, it seemed likely that this approach was of limited value in the analysis of differential cross reactions.

5.4 Agglutination assays

Agglutination tests involved the use of high concentrations of antigen and antibody. In this study the agglutinating property of the MCAbs were tested under different conditions.

5.4.1 Slide agglutination tests

The ammonium sulphate concentrated MCAbs (Section 2.3.4.3) or ascitic fluid were added to equal volumes of suspensions of gram-negative bacteria on a slide. The results obtained are presented in the Table 5.3. The three MCAbs that were positive to Inaba on ELISA (Section 3.6) agglutinated

Fig. 5.3 Effect of more and less stringent washing on ELISA signal

MCAb I4C3 was reacted with Ogawa or Inaba vibrios on ELISA and was subjected to varying wash protocols.

A ELISA plates washed with PBS, two times

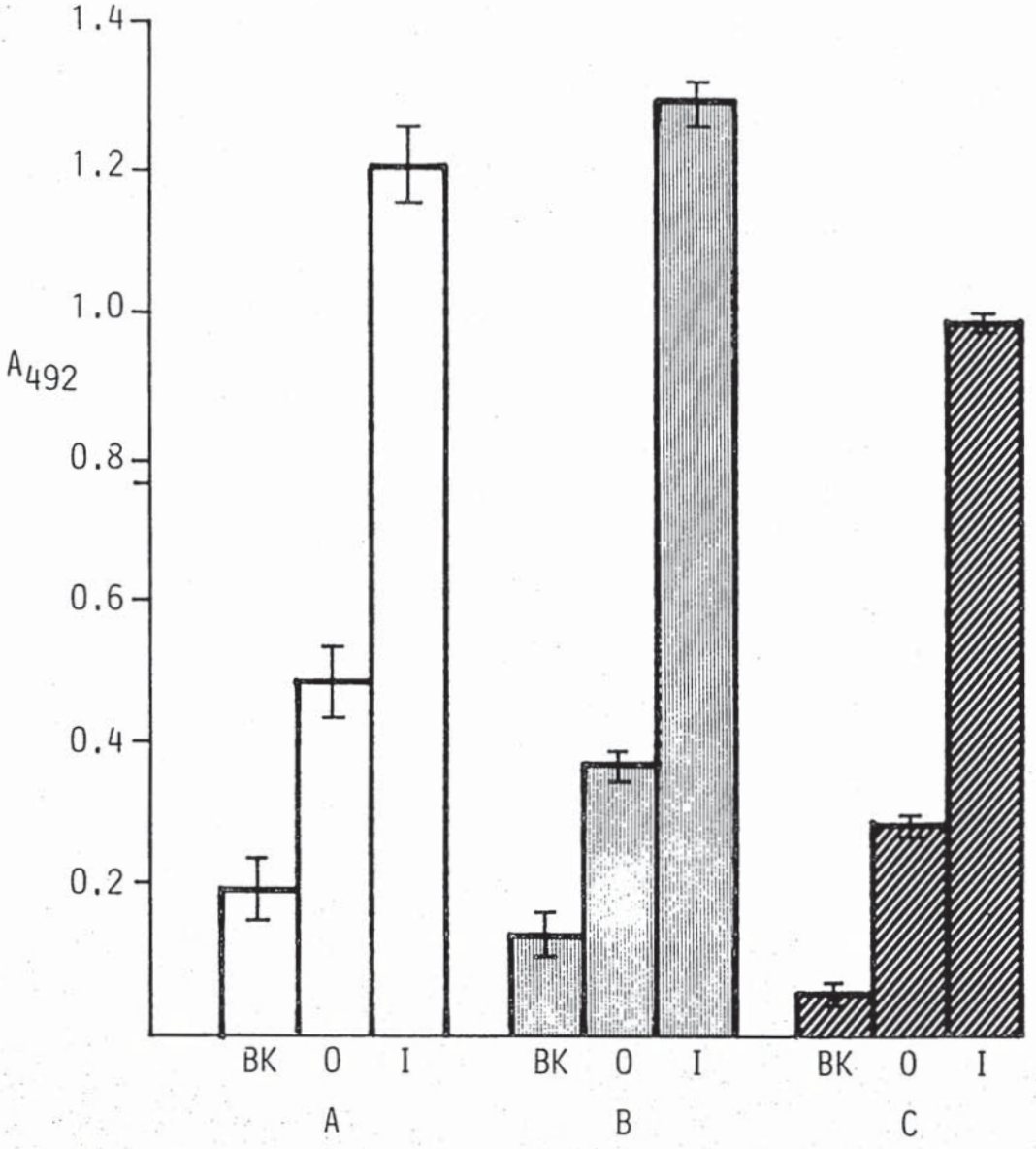
B ELISA plates washed with PBS-Tween, two times

C ELISA plates washed with PBS-Tween, six times

Bk ELISA plates coated with no antigen

O ELISA plates coated with Ogawa

I ELISA plates coated with Inaba


1.4
1.2
1.0
A492
0.8
0.6
0.4
0.2
BK
O
I
A
BK
O
I
B
BK
O
I
C

Wash Buffer	No. of washes	Absorbance at 492nm		Ratio of
		Ogawa	Inaba	Ogawa/Inaba
PBS	2	0.29	1.01	0.29
PBS-Tween	2	0.24	1.16	0.21
PBS-Tween	6	0.23	0.93	0.25

Table. 5.2 Effect of washing on background reaction of ELISA

I4C3 antibody was reacted with Inaba vibrios and the ELISA plate was washed by different methods.

Table 5.3 Slide agglutination with MCAbs

Agglutination tests were performed as described in Section 2.3.3.4.2.

+++	Formation of large clumps within a minute
++	Formation of small clumps within a minute
+	Formation of small clumps in two minutes
-	No agglutination

Bacteria	Score of Monoclonal Antibodies						Score of Ascitic fluid	
	I1A1	I4B1	I4C3	O4A6	O4D2	O3D1	O4A6	I4B1
Ogawa	+++	+++	+++	-	-	-	-	+++
Inaba	+++	+++	+++	-	-	+	-	+++
B.abortus	+++	+++	+++	-	-	-	-	+++
B.melitensis	-	-	-	-	-	-	-	-
E.coli	-	-	-	-	-	-	-	-
Y.enterocolotica	-	-	-	-	-	-	-	-

Ogawa vibrios and Brucella abortus organisms with the same efficiency as they agglutinated the Inaba vibrios. O3D1, showed a slight reaction with Inaba vibrios and no other agglutination characteristics.

5.4.2 Agglutination in V-bottom plates

The agglutination tests performed on the slides were not quantitated to give an indication of the relative affinity of the antibodies for the variety of antigens used. For the easy quantitation of the agglutination data the tests were carried out in V-bottom plates as described in (Section 2.3.3.4.3).

5.4.2.1 Primary agglutination test

The MCAbs were sequentially double diluted starting with the 20 times concentrated antibody. Each dilution of the antibody was added to an equal volume of either Ogawa or Inaba vibrios in to the V-bottom wells. The agglutination was scored as in Table 5.4.

The MCAbs raised against the Ogawa serotype did not exhibit good agglutinating properties. The agglutination produced by O3D1 was weak but it indicated a definite preference for the Inaba serotype in all tests with different batches of antibody and antigen. The anti-Inaba MCAbs, on the other hand, agglutinated both the serotypes of V. cholerae. The strength of the agglutinating properties of the antibodies were estimated by the lowest dilution of the antibody (titre) capable of producing agglutination. All three Inaba positive MCAbs (ELISA)

Table 5.4 Scores of primary agglutination

Agglutinations were performed as described in Section 2.3.3.4.3 and the intensity of agglutination was scored visually by the area of the mat formed. MCAbs were sequentially double diluted starting with a concentration of 20 times neat culture supernatant.

+++	Large mat
++	Medium mat
+	Small mat
±	Small mat and a pellet
-	only a pellet, no agglutination

Monoclonal Antibody	Score and endpoint titre against		Ratio of endpoint titre Inaba/Ogawa
	Ogawa	Inaba	
O4A6	± 5	-	n.a
O4D2	± 20	-	n.a
O3D1	+ 5	+ 2.5	1/2
I1A1	+++ 2.5	+++ 1.25	1/2
I4B1	+++ 1.25	+++ 0.6	1/2
I4C3	+++ 1.25	+++ 0.3	1/2

Fig. 5.4 Secondary Agglutination in V-Bottom plates

All the odd numbered wells contained $5x10^9$ Ogawa vibrios, 10ul of 1 in 10 rabbit anti-rat IgG and different MCAbs. All the even numbered wells contained $5x10^9$ Inaba vibrios, 10ul of 1 in 10 rabbit anti-rat IgG and different MCAbs.

Columns 1-2 MCAb 03D1

Columns 3-4 MCAb 04A6

Columns 5-6 MCAb 04D2

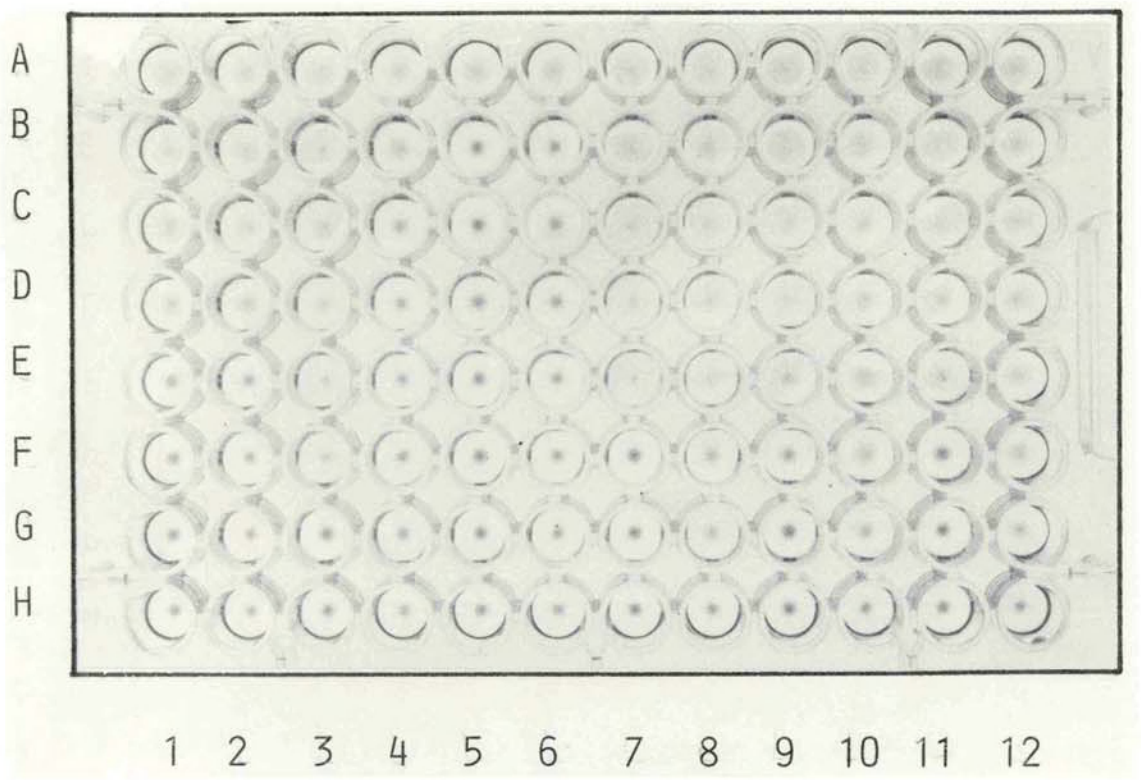
Columns 7-8 MCAb I1A1

Columns 9-10 MCAb I4B1

Columns 11-12 MCAb I4C3

Row A-G Doubling dilution of each MCAb starting with 20 times concentrated antibody

Row H Control, MCAb substituted with equal volume of PBS


A
B
C
D
E
F
G
H
1 2 3 4 5 6 7 8 9 10 11 12

had a higher titre for the Inaba serotype than for the Ogawa serotype, as did O3D1. These data confirmed the cross reactivity detected by the IF assay and produced a new cross reaction in that of O3D1 which had been entirely serotype specific in other assays.

5.4.2.2 Secondary agglutination

In the secondary agglutination assay, a second antibody, the rabbit anti-rat antiserum was added to enhance the formation of a network for agglutination. Monoclonal antibodies, particularly IgG ones, can be anticipated to be highly variable reagents for cross linking organisms because their single epitope nature may have very precise geometric requirements which cannot be simulateneously satisfied by both organisms to be agglutinated. The addition of a second, polyclonal reagent directed against the first may therefore be expected to give better agglutination in general and, in a case such as this, potentially alter the specificity profile.

The addition of the anti-rat serum helped O4A6 but not O4D2 to agglutinate the Ogawa vibrios (Table 5.5). For the I1A1 and I4B1 antibodies, the preference for the Inaba serotype was increased as demonstrated by the ratio of the end point titres against the two serotypes while I4C3 remained unaffected (Table 5.5). Generally, the titres for the agglutination were increased in the presence of the second antibody. An interesting feature of the second antibody was that the preference for the heterologous serotype by O3D1 was not observed.

Table 5.5 Scores of secondary agglutination

MCAbs were tested for agglutination in presence of a second antibody, rabbit anti-rat IgG. The antibodies were sequentially double diluted starting with 20 times concentrated solution. Agglutinations were scored visually by the area of mat formed.

+++	Large mat
++	Medium mat
+	Small mat
±	Small mat and a pellet
-	Only a pellet, no agglutination

Monoclonal antibody	Score and endpoint titre		Ratio of end point titre Inaba/Ogawa
	Ogawa	Inaba	
O4A6	++ 0.6	± 10	16.7
O4D2	± 20	± 20	1
O3D1	++ 2.5	++ 2.5	1
I1A1	+++ 1.25	+++ 0.3	1/4
I4B1	+++ 1.25	+++ 0.3	1/4
I4C3	+++ 1.25	+++ 0.3	1/4

5.4.3 Slide agglutination with staphylococcal protein A-adsorbed antibody

Many routine clinical serotyping assays involve the use of antibody adsorbed to Staphylococcus aureus as an agglutination test as the local concentration of antibody can be greatly increased by binding to the surface of the S. aureus. This technique is claimed to increase the sensitivity of agglutination assays and is generally employed with rabbit polyclonal antisera.

MCAbs were adsorbed to Staphylococcus aureus, strain Cowan I, as described by Kronvall (1973), (Section 2.3.3.4.2). During the process of coating the staphylococci with the MCAbs it was observed that the staphylococci organisms themselves were agglutinated by the antibodies. Normal rat serum used as a control also agglutinated the organisms. The batch of staphylococci used had been stored for frozen for two years. A fresh batch of staphylococci was tried and yielded the same results.

The techniques employing S. aureus-coated antibodies were established for rabbit antibodies which bind well and specifically to the protein A of the organism. Rat antibodies bind poorly to the protein A of S. aureus (Rousseaux et al, 1981; Nilsson et al, 1982) and hence may not be suited to the assay. The fact that normal serum from several rats also gave high controls made the assay of little value and this approach was taken no further.

5.4.4 Primary Agglutination at altered pH values

Mossman et al, (1980) reported the alteration of the specificity of MCAbs with a change in the pH of the buffer in which the assay was performed. As Ogawa LPS has been reported to have the additional sugar 4,amino-arabinose and at least two of the originally defined Ogawa panel of MCAbs were totally specific, it was logical to see if pH could affect this specificity, and in particular, if the use of a high pH close to the expected pK of the specific sugar group, would alter the specificity profile of these antibodies. The high cross reaction of I1A1, I4B1 and the I4C3 and the mild cross reaction of the O3D1 were therefore tested on vibrios in acidic and alkaline buffers (Section 2.3.3.4.3). In the process of washing the vibrios in an acid and an alkaline buffer the texture of the suspension changed and this made comparison of the agglutination data at different pH values difficult.

There was no change in the titres of O4A6 and O4D2 with respect to their weak ability to agglutinate the vibrios at the selected pH values (Table 5.6). There was a drop in the titre of O3D1, I4B1 and I4C3 for both the serotypes at both the alkaline and the acidic pH values (Table5.6). Thus significant changes in the specifity of the MCAbs were not observed at altered pH. At pH values below 6 the vibrios formed clumps which were difficult to disperse, and consequently, a lower pH was not used for the test.

Table 5.6 Agglutination at different pH

20 times concentrated MCAbs were tested for agglutination at different pH values as described in Section 2.3.3.4.4.

- No agglutination

Monoclonal Antibody	Endpoint titre agglutination with			Endpoint titre agglutination with		
	Ogawa			Inaba		
	pH 6	pH 7.6	pH 9.2	pH 6	pH 7.6	pH 9.2
O4A6	10	5	-	20	-	20
O4D2	20	20	-	20	-	20
O3D1	20	5	-	10	2.5	10
I1A1	1.25	2.5	5	1.25	1.25	1.25
I4B1	1.25	1.25	2.5	1.25	1.25	2.5
I4C3	1.25	1.25	5	1.25	1.25	1.25

5.5 The effect of high antigen concentration on the specificity of the MCAbs on ELISA

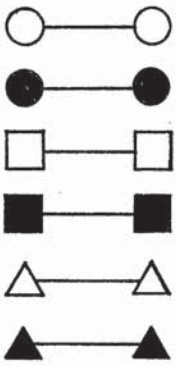
The cross-reactivities of some of the MCAbs was clearly exhibited in the agglutination assays (Section 5.4). The apparently Inaba specific MCAbs cross-reacted not only with the heterologous serotype but also Brucella abortus in the agglutination tests (Table 5.3). The similarity between V. cholerae LPS and B. abortus LPS was reported earlier by Feeley (1969) from agglutination studies conducted with rabbit antisera. The validity of the agglutination data obtained in Section 5.4 were checked on ELISA by coating the plates with a logarithmic increase in antigen concentration.

5.5.1 The specificities of the apparently Ogawa specific antibodies

04A6, 03D1 and 04D2 were reacted with increasing amounts of vibrios of either serotype. In cases where the absorption was beyond the scale of the spectrophotometer, the curve was extrapolated at a value of 2.0 O.D. units although the actual reading was immeasureably greater. It was observed that all three MCAbs had high activities against Ogawa when large numbers of the vibrios were coated onto the ELISA plates (Fig. 5.5a). However, the increase in the antigen-antibody binding levelled off at 10^7 vibrios on the plate. This indicated that the antibody concentration was limiting beyond 10^7 vibrios per well. The binding efficiency decreased by 50% with the fall in antigen

Fig.5.5 a Activity of MCAbs on increased antigen density

Increasing number of Ogawa or Inaba vibrios were coated on ELISA plate and MCAbs were reacted with them. The values plotted are a mean obtained form three assays.



O4A6 reacted with Ogawa vibrios

O4A6 reacted with Inaba vibrios

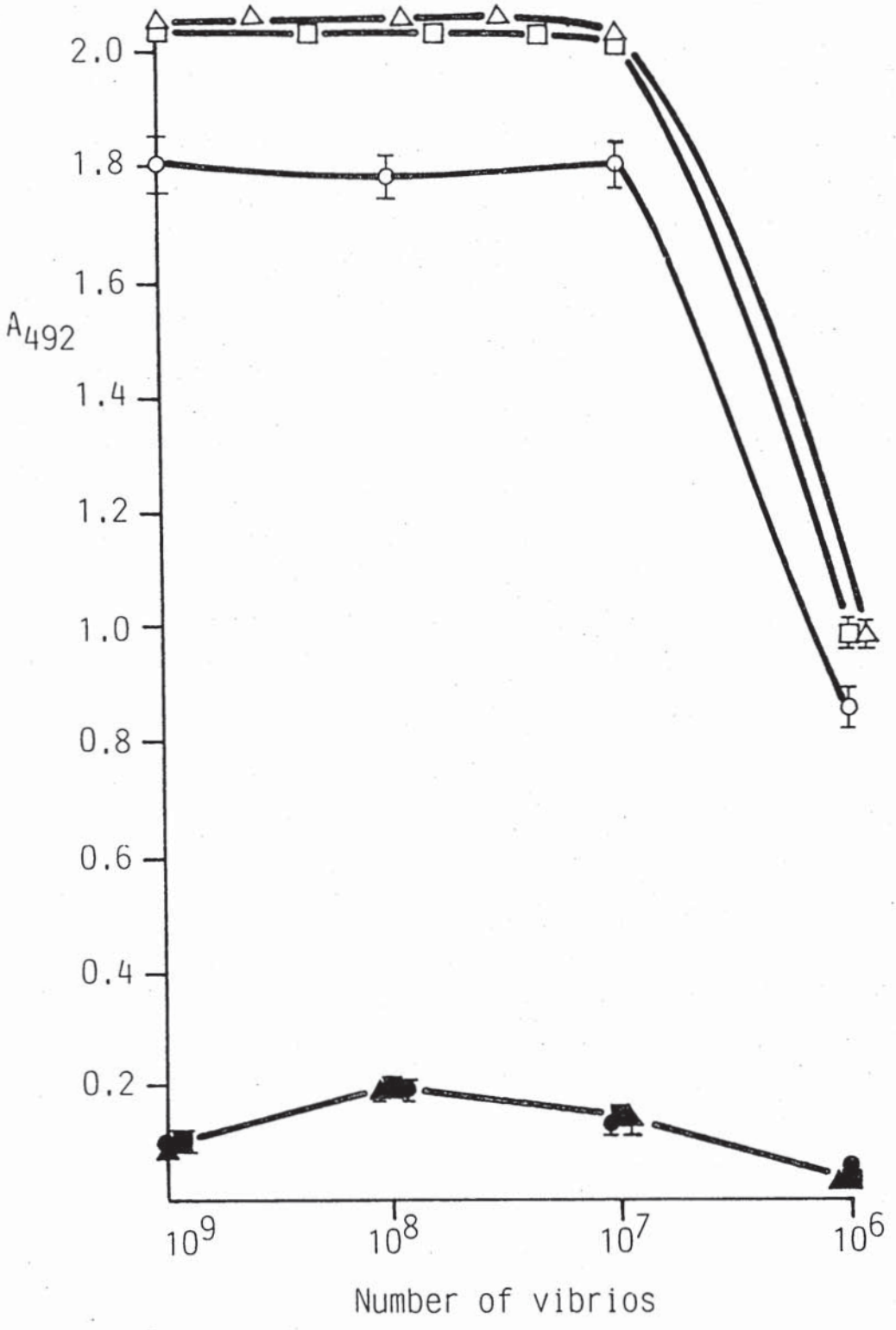
O3D1 reacted with Ogawa vibrios

O3D1 reacted with Inaba vibrios

O4D2 reacted with Ogawa vibrios

O4D2 reacted with Inaba vibrios

Background reaction was below 0.1 O.D.


2.0
1.8
1.6
A492
1.4
1.2
1.0
0.8
0.6
0.4
0.2
10^9
10^8
10^7
10^6
Number of vibrios

concentration from 10^7 to 10^6 vibrios per well.

When O4A6, O3D1 and O4D2 were reacted with increasing amounts of Inaba vibrios, they exhibited a different pattern. At the maximum concentration of the antigen coated on the plate, 10^9 vibrios per well, no binding was observed. A weak positive reaction was detected at 10^8 vibrios per well. This dropped to half the value at 10^7 per well, and disappeared below the background at 10^6 per well. The highest binding activity of the MCAbs to Inaba was about one tenth of that of that towards Ogawa.

The MCAbs were also reacted with similar concentrations of B. abortus, B. melitensis and E. coli. There was no significant reaction of the antibodies against these bacteria (Fig. 5.5b). The rabbit anti-rat conjugate (Miles) gave a relatively high background with the B. melitensis organisms.

5.5.2 The specificities of the anti-Inaba MCAbs

The anti-Inaba MCAbs, I1A1, I4B1 and I4C3 showed strong reaction with Inaba vibrios (Fig. 5.5c). This panel of antibodies showed concentration limitations at the same level as the Ogawa panel, i.e. 10^7 per well. However, the activities at vibrio concentration of 10^6 were comparatively high.

At concentrations of 10^9 Ogawa vibrios per well, the anti-Inaba MCAbs had little activity. However, there was an increase in antibody binding at 10^8 per well which reached its maximum at 10^7 per well. It then fell sharply to background values at 10^6 per well (Fig. 5.5c). The activity of the

Fig.5.5 b Activity of MCAbs on increased antigen density

Increasing number of various bacteria were coated on ELISA plate and MCAbs were reacted with them. The values plotted are a mean obtained form three assays.

Mean activity of all six MCAbs on B. abortus

Mean activity of all six MCAbs on B. melitensis

Mean activity of all six MCAbs on E. coli

Background reaction was 0.15 O.D.

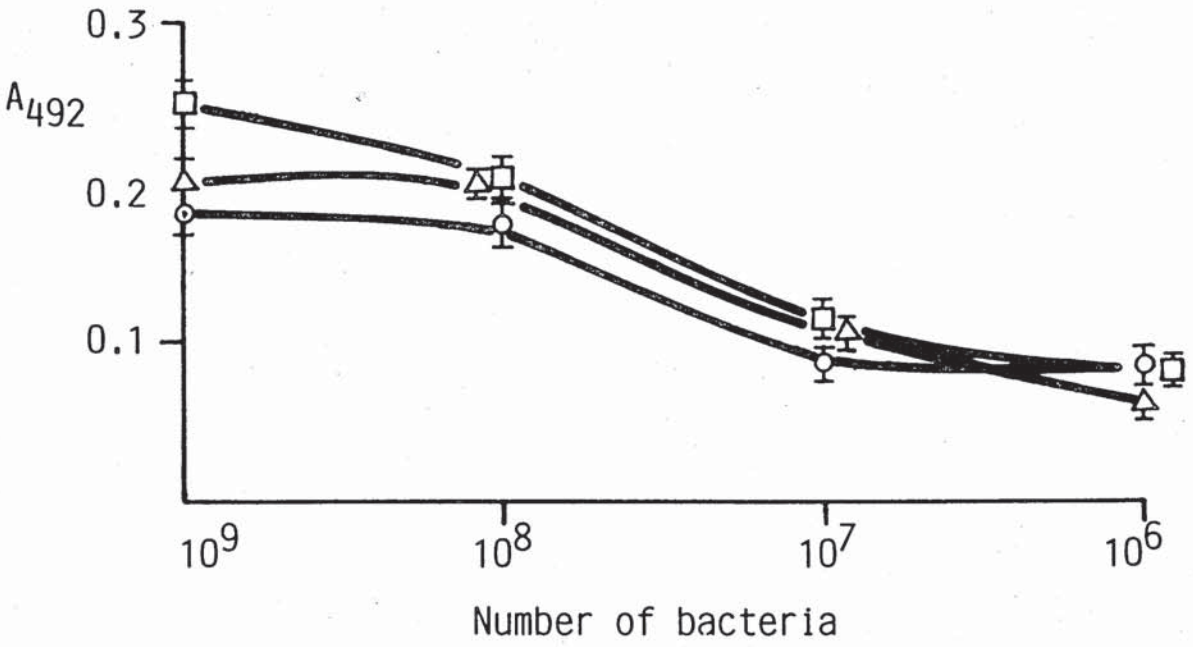
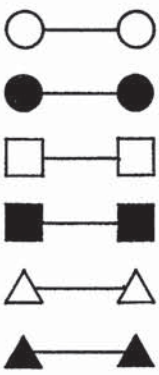

0.3
A_{492}
0.2
0.1
10^9
10^8
10^7
10^6
Number of bacteria

Fig. 5.5c Activity of MCAbs on increased antigen density

Increasing number of Ogawa or Inaba vibrios were coated on ELISA plate and MCAbs were reacted with them. The values plotted are a mean obtained form three assays.



I1A1 reacted with Ogawa vibrios

I1A1 reacted with Inaba vibrios

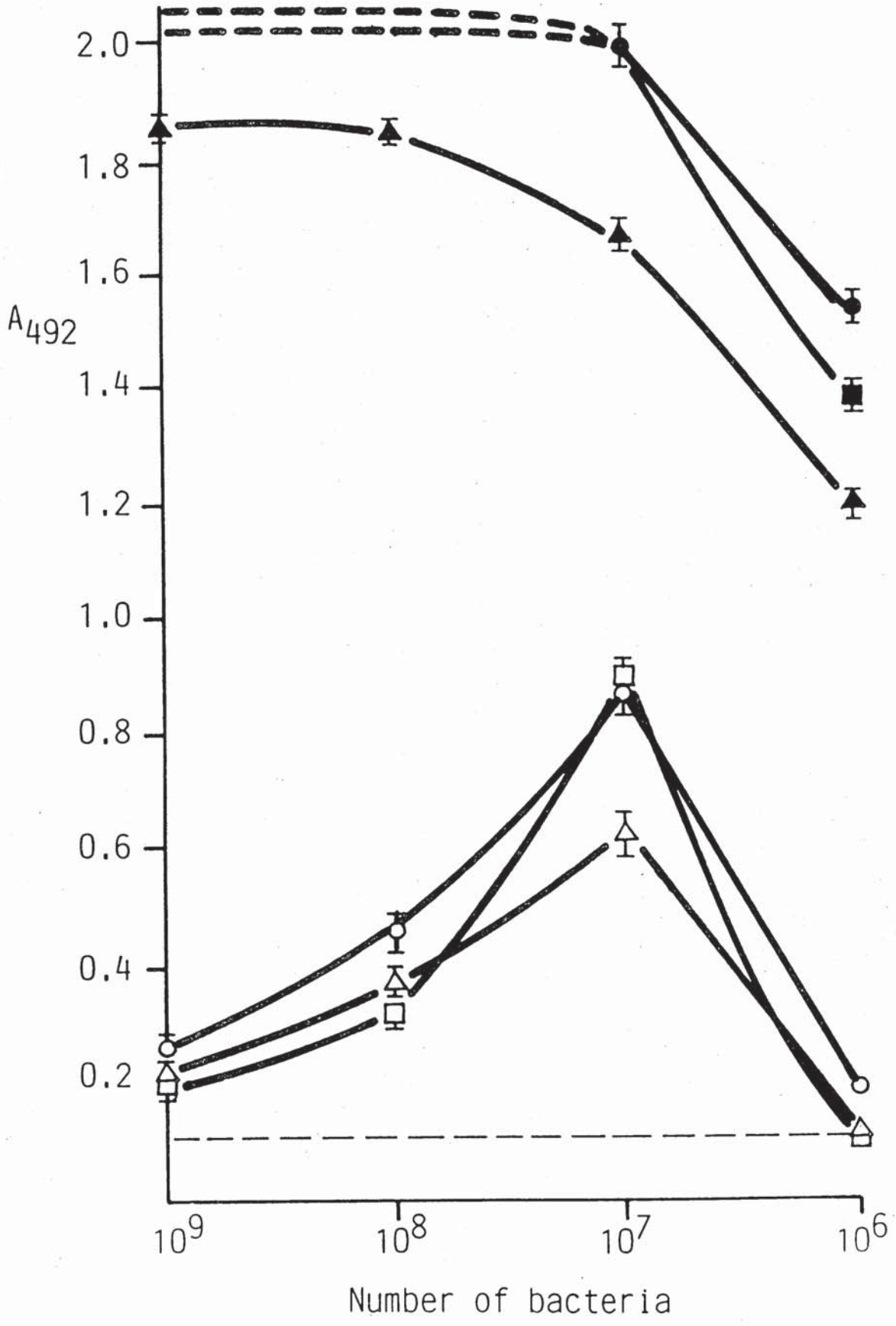
I4B1 reacted with Ogawa vibrios

I4B1 reacted with Inaba vibrios

I4C3 reacted with Ogawa vibrios

I4C3 reacted with Inaba vibrios

Background reaction was below 0.1 O.D.


2.0
1.8
1.6
A492
1.4
1.2
1.0
0.8
0.6
0.4
0.2
10^9
10^8
10^7
10^6
Number of bacteria

anti-Inaba MCAbs at its maximum against Ogawa was half of that against Inaba.

The reaction of the antibodies against B. abortus, B. melitensis and E. coli(ML 308) were very similar to that of the anti-Ogawa MCAbs (Fig. 5.5 b).

5.6 Heterologous activity of concentrated O4A6 and O3D1 antibodies

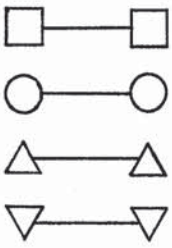
The earlier ELISA results, competition ELISA results and immunoblotting data clearly indicated that O4A6, O3D1 and O4D2 had negligible activity against the Inaba serotype vibrios. This was in agreement with the indirect IF assay and the agglutination assays with the exception of O3D1 which showed an unexpected preference for the heterologous serotype (Section 5.4.4). If a low affinity interaction with the heterologous serotype by anti-Ogawa antibodies such as by O3D1 is a genuine observation (Section 5.4.1), then such a reaction might be enhanced at higher concentrations of the antibody. Consequently, large amounts of O3D1 and O4D2 obtained from ammonium sulphate precipitation were tested on increased quantities of antigen to detect any binding with Inaba vibrios on ELISA.

When large quantitites of O3D1 and O4A6 were reacted with increasing numbers of Ogawa vibrios, the binding of the antibodies increased till it reached a maximum value at a much higher concentration of the antigen on the plate (Fig. 5.6). This was in contrast to the results observed in Section 5.5.1 where the antibody was limiting. Even at the high concentrations

Fig.5.6 Effect of increased antibody concentration on increasing antigen density

Ten times concentrated MCAbs O4A6 and O3D1 were reacted with increasing numbers of Ogawa and Inaba vibrios.

The values plotted are a mean of two assays.



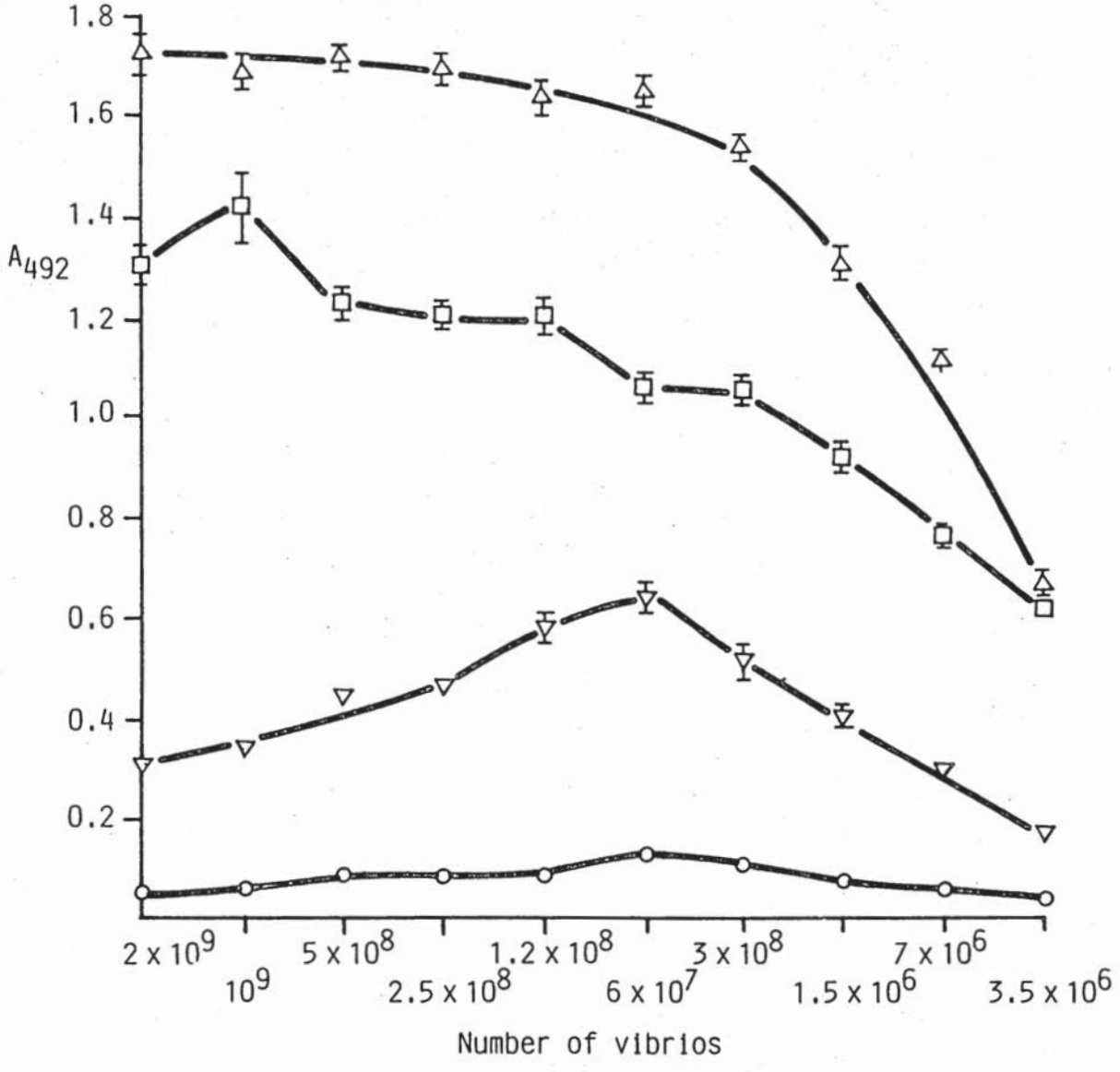
O4A6 reacted with Ogawa vibrios

O4A6 reacted with Inaba vibrios

O3D1 reacted with Ogawa vibrios

O3D1 reacted with Inaba vibrios

Background reaction was below 0.10.D.


1.8
1.6
1.4
A492
1.2
1.0
0.8
0.6
0.4
0.2
2 x 10^9
10^9
5 x 10^8
2.5 x 10^8
1.2 x 10^8
6 x 10^7
3 x 10^8
1.5 x 10^6
7 x 10^6
3.5 x 10^6
Number of vibrios

of antibody used for the assay O4A6 did not react significantly with Inaba vibrios. O3D1 showed a different pattern, binding significantly to Inaba (Fig.5.6), and demonstrating significant cross-reaction. Since only O3D1 reacted at high concentrations with the Inaba it was clear that the other anti-Ogawa antibody did not have demonstrable anti-Inaba activities on this assay.

The skewed distribution in the ELISA reaction of the MCAbs to increasing amounts of heterologous antigen was again observed with O3D1 (Fig. 5.6).

5.7 Variation in ELISA activity at increasing amounts of antigen

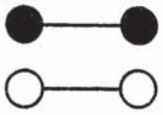
The skewed distribution of activity on ELISA of I1A1, I4B1 and I4C3 against Ogawa vibrios (Fig. 5.5 c) and ten times O3D1 against Inaba vibrios (Fig. 5.6) may be due to various factors. The leaching effect of the antigen at very high concentrations may result in some primary antibody being washed off the plate with antigen (Frankel and Gerhard, 1979). However, one would also expect to observe this with homologous vibrios. It is possible that such an effect is occurring with the homologous virbrios but is poorly resolved at high optical density readings. Optical density is measured on a logarithmic scale and the measurements are poorly resolved above values of 1.5 so that printouts in this range from the Titretrek Multiskan may lead to data which is of limited accuracy. It is however, very possible that the skewed effect is due to zone effects at high antigen concentrations with the enzyme labelled second antibody having limited access. In this context, it is interesting to note that

in direct binding assays utilising labelled monoclonals, skewed distribution of this type was not detected over the same antigen concentration range (Figure 5.13).

5.8 Complement fixation by anti-V. cholerae MCAbs

The ability of the panel of monoclonal antibodies to fix complement was tested for several reasons. Firstly, the micro-complement fixation assay of Wasserman and Levine, 1961 could be carried out at more physiological concentrations of antibody and antigen than an agglutination assay which is conducted at artificially high concentrations and is consequently frequently suggested to be misleading due to "non-specific" interactions. Complement fixation is, however, also an assay that does not involve washing procedures and may therefore detect low affinity reactions, difficult to detect in ELISA and IF and so the general specificity profile of the panel on this assay could be expected to provide additional information on the relative contribution of specificity and affinity to cross reactions among monoclonal antibodies. In addition, few rat monoclonal antibodies (Clark et al, 1983) and no IgG2b rat monoclonal antibodies, have been tested for their ability to activate the complement pathways. The ability of the anti-V. cholerae MCAbs raised in this study to fix complement against V. cholerae was tested with whole vibrios as described in Section 2.3.3.5. Fig. 5.7 illustrates the concentrations of the antibodies which could be demonstrated to fix complement against either serotype

Fig. 5.7 a and b Complement fixation activity of MCAbs

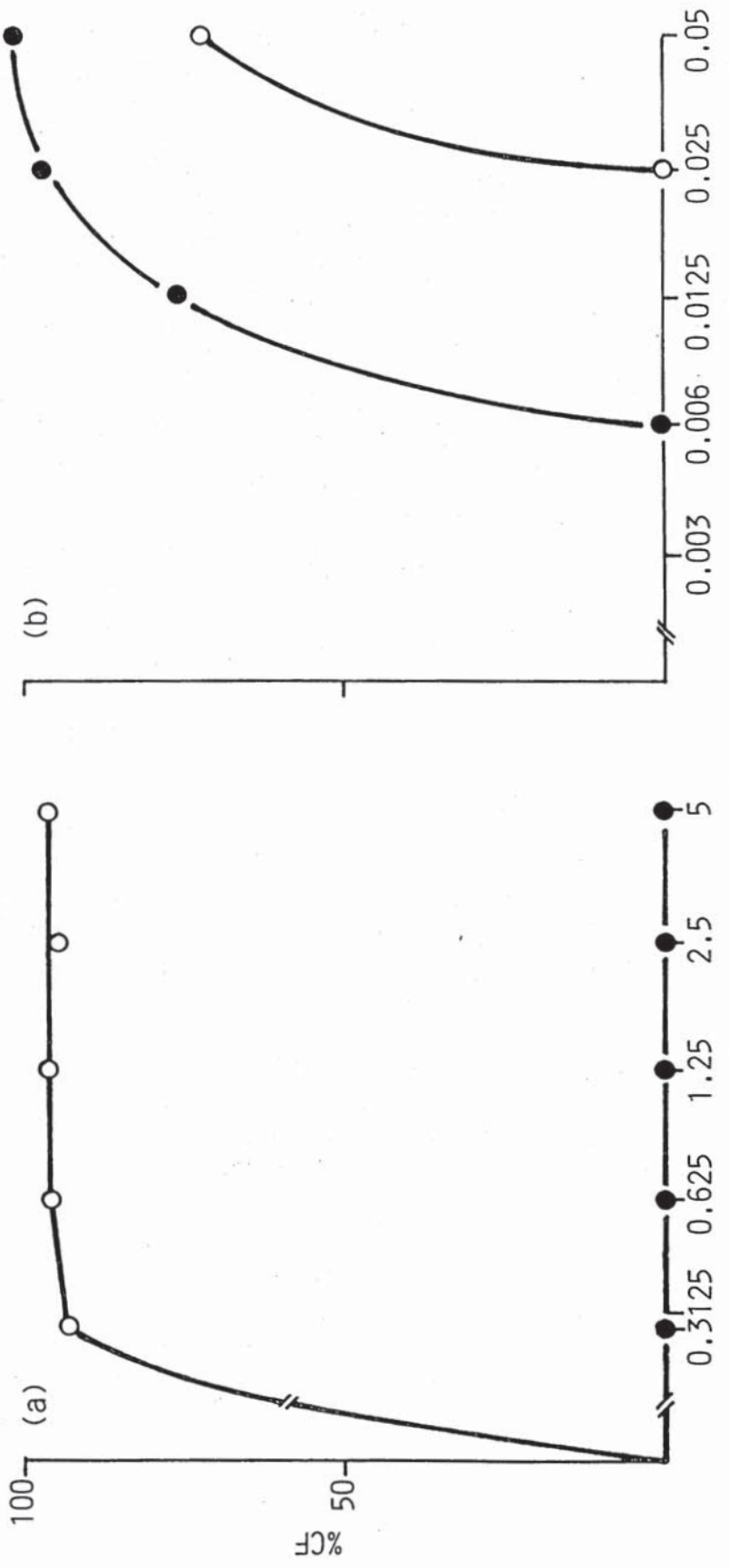


Activity against Inaba vibrios

Activity against Ogawa vibrios

a) CF activity of O4A6

b) CF activity of I1A1


100
50
%CF
(a)
0.3125
0.625
1.25
2.5
5
Dilution of MCAb 04A6
(b)
0.003
0.006
0.0125
0.025
0.05
Dilution of MCAb I1A1

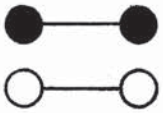
of V. cholerae. O4A6 and O4D2 exhibited monospecificity towards the Ogawa serotype. O4D2 fixed 100% complement against Ogawa at a dilution of 0.5 and none against Inaba at the same dilution. O4A6 was tested at five times its strength in culture medium. Complement was not fixed by the high amounts of the antibody against Inaba and above 90% of complement was fixed against Ogawa.

O3D1, the antibody which showed unexpected cross reaction on agglutination, was tested over a wide range of antibody concentrations against both Ogawa and Inaba vibrios. At five its strength in culture medium O3D1 was able to fix 100% complement against both Ogawa and Inaba. The percentage of complement fixed against Inaba dropped as the antibody was diluted. It could no longer fix any complement against the Inaba vibrios at the dilution of 0.63, however, there was 100% complement fixation at dilutions as low as 0.05 against the Ogawa vibrios. This confirms the qualitative result of the agglutination assay although the quantitative results are different.

I1A1, I4B1 and I4C3 showed cross-reaction with Inaba and Ogawa to the same extent, though high amounts of antibody I4B1 were required for the demonstration of complement fixation. The weak reaction of I4B1 was interesting but may relate to the fact that the ELISA titre of this antibody in this particular assay was low.

The cross-reactivity of the anti-Inaba MCAbs with B. abortus on agglutination assays was confirmed on complement

Fig. 5.7 c and d Complement fixation activity of MCAbs



Activity against Inaba vibrios

Activity against Ogawa vibrios

a) CF activity of O4D2

b) CF activity of I4B1

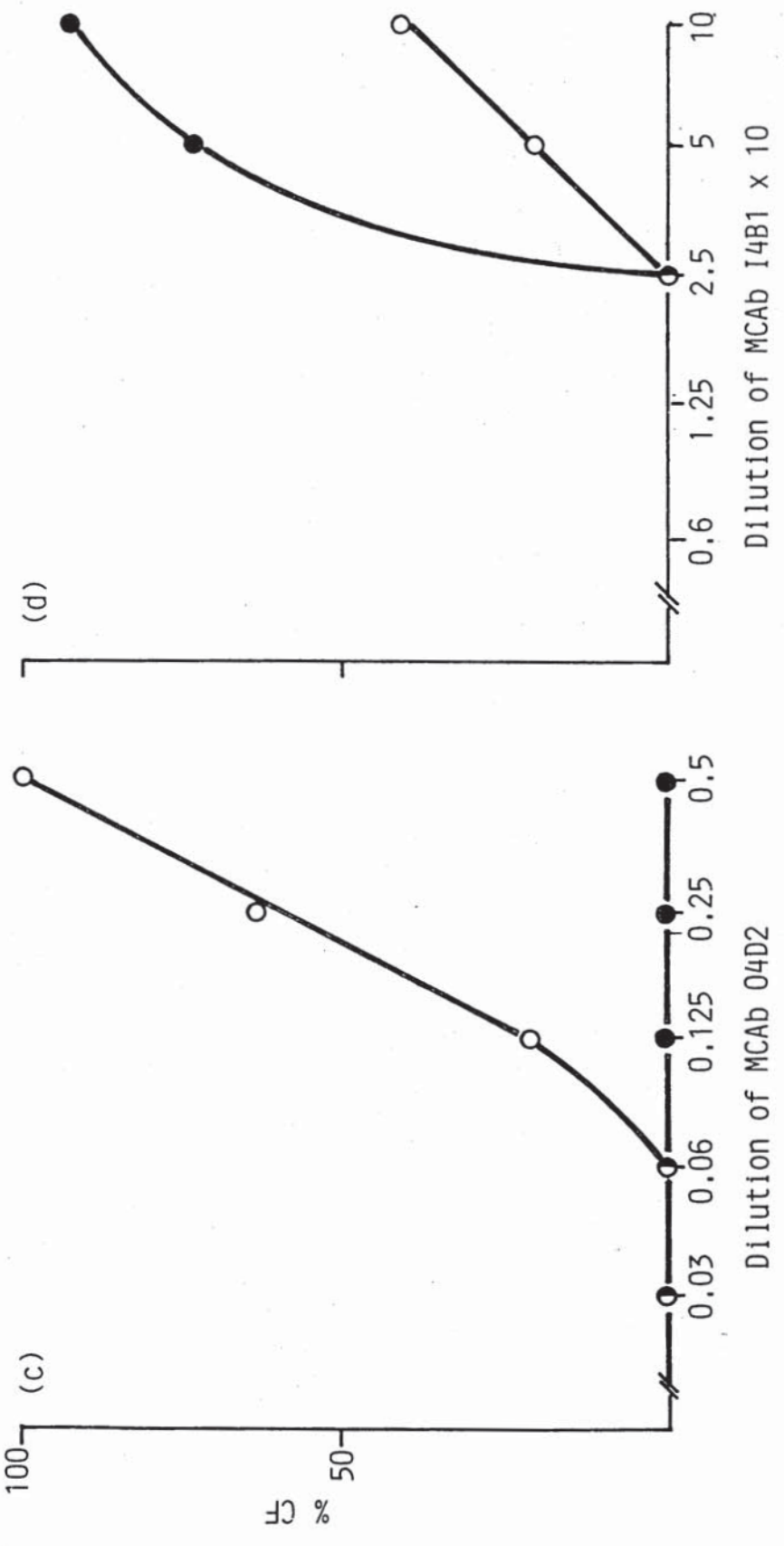
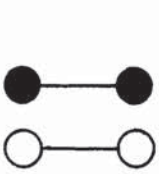
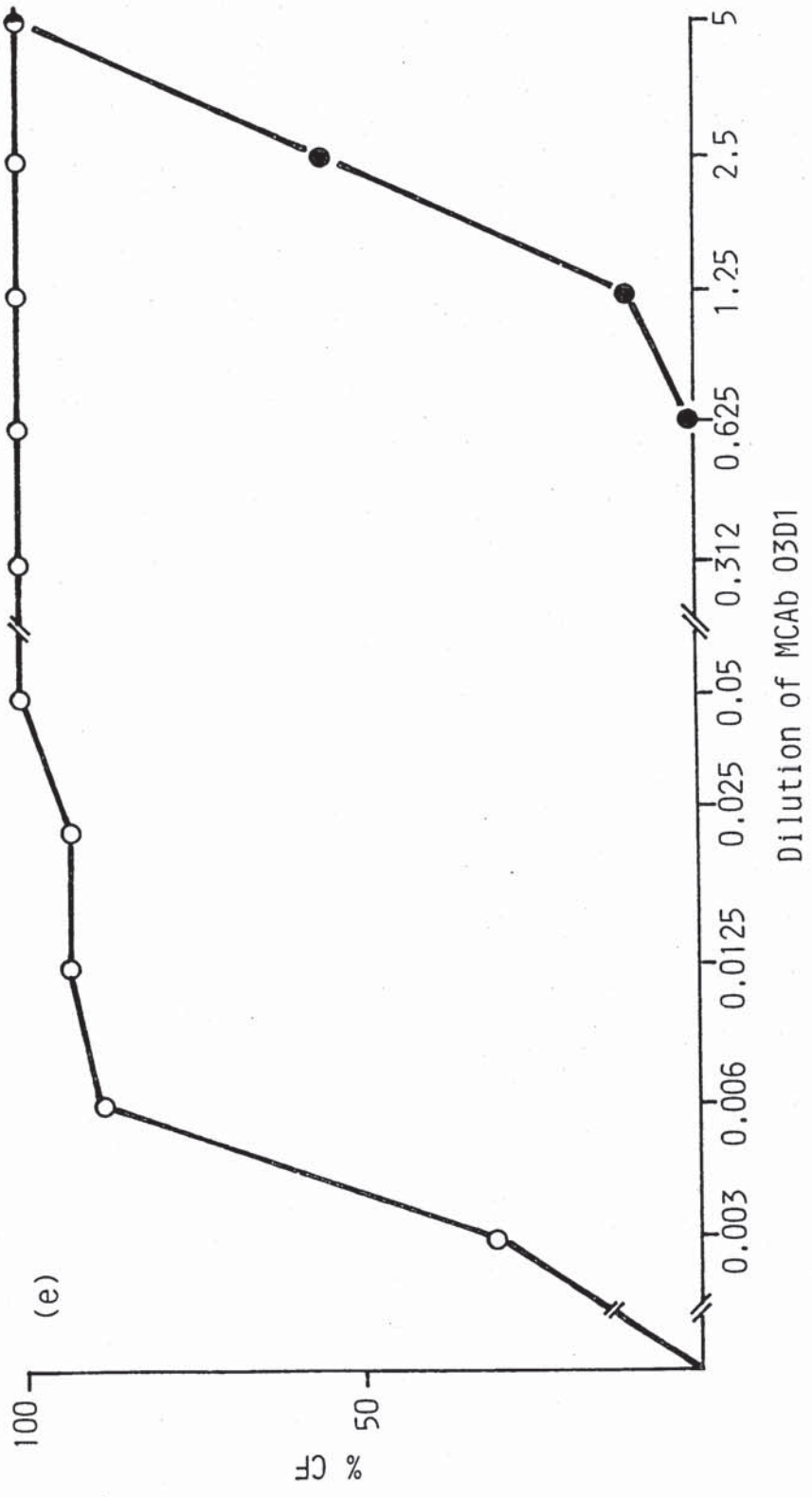

(c)
100
50
% CF
0.03
0.06
0.125
0.25
0.5
Dilution of MCAb 04D2
(d)
0.6
1.25
2.5
5
10
Dilution of MCAb I4B1 x 10

Fig. 5.7 e Complement fixation activity of MCAb




(e)
100
50
% CF
0.003
0.006
0.0125
0.025
0.05
0.312
0.625
1.25
2.5
5
Dilution of MCAb 03D1

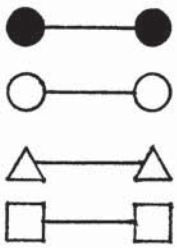
fixation assay with I4C3. The antibody fixed complement against B. abortus to a lesser extent than it did with Inaba or Ogawa vibrios (Fig. 5.7 f). I1A1 and I4B1 were not tested in this assay.

Various gram negative bacteria or their LPS have been reported to fix complement by classical pathways in the absence of antibodies (Grossman and Leive, 1984; Joiner et al, 1984; Wilson and Morrison, 1982). However, no fixation was observed with the control tubes containing vibrios alone indicating that either V. cholerae LPS does not fix complement, or that the relevant chemical structures of the LPS were not accessible to the complement pathway on the whole vibrios.

5.9 Cross-reactivity of the MCAbs

The apparent serotype specificity of the MCAbs observed in the ELISA (Section 3.6), the Western blots (Section 4.8) and the competition ELISA (Section 5.1) were thus not observed in all the other assays described in this chapter. The Inaba specific antibodies cross-reacted with the Ogawa vibrios, though they generally exhibited a preference for the Inaba antigen, and O3D1 showed some cross reaction with Inaba vibrios. It was clearly of relevance to determine whether these antibodies detected identical epitopes or overlapping epitopes on the two serotypes or whether each antibody recognised a distinct region of the LPS.

Fig.5.7 f Complement fixation activity of MCAbs



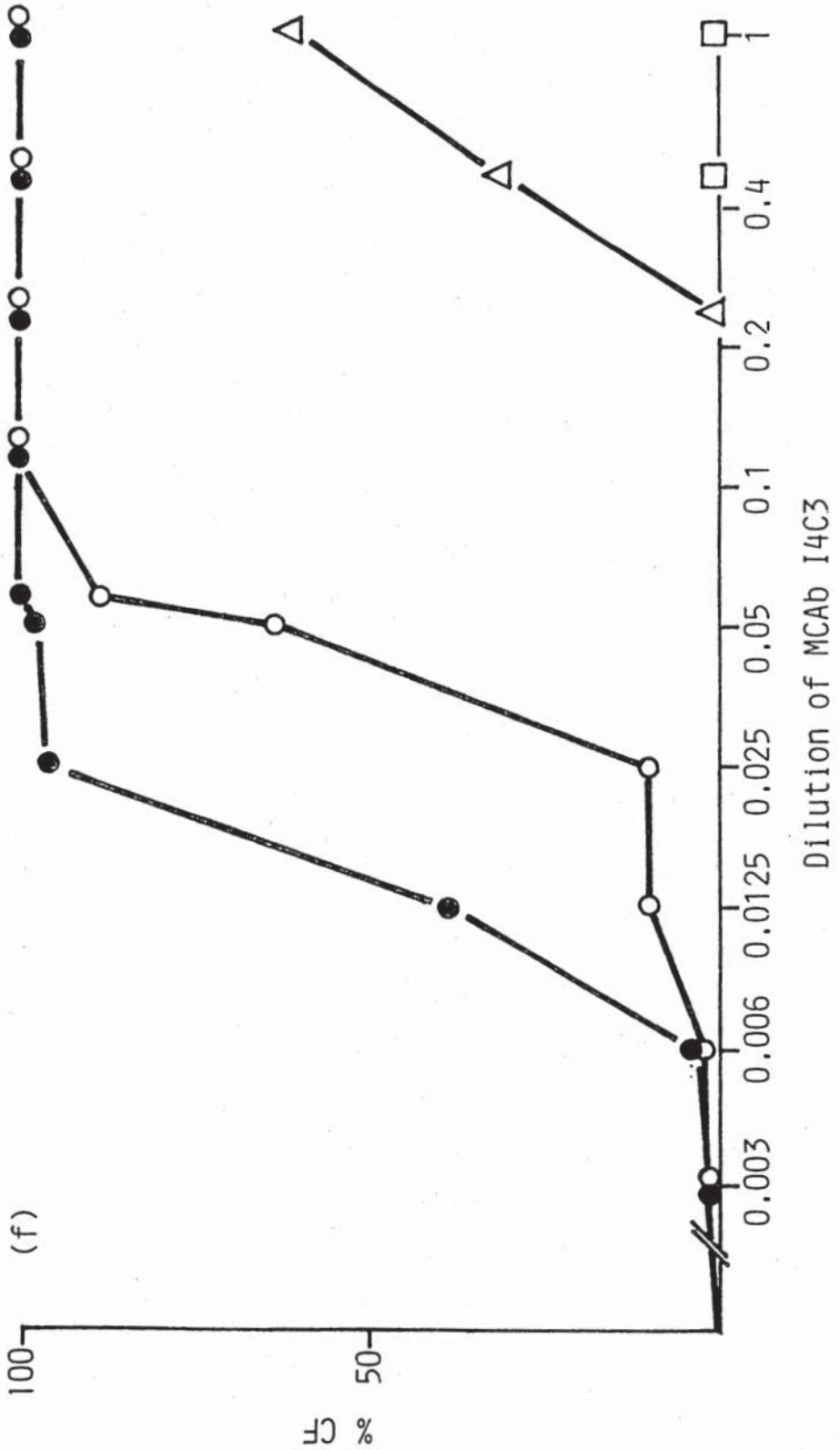
CF activity of I4C3

Activity against Inaba

Activity against Ogawa

Activity against B. abortus

Activity against B. melitensis


(f)
100
50
% CF
0.003
0.006
0.0125
0.025
0.05
0.1
0.2
0.4
1
Dilution of MCAb I4C3

5.10 Detection of epitopes on V. cholerae LPS by ELISA

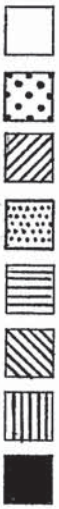
Friguet et al (1983) have described an additive ELISA method by which it was possible to demonstrate that two separate MCAbs detected two different epitopes on the same antigen. The same principle was applied to investigate the epitopes recognised by the anti-Inaba antibodies on the Ogawa LPS. The Ogawa vibrios were preincubated with saturating amounts of either of the Ogawa specific antibodies to block the sites recognised by them and washed. The addition of either I1A1, I4B1 or I4C3 antibodies to the blocked vibrios should have increased the binding of the peroxidase conjugated anti-rat serum to the plate if any of the anti-Inaba antibodies had bound to any site which was distinct from that recognised by O4A6, O4D2 and O3D1. There was no increase in the absorbance after the double incubations of various combinations of antibodies as illustrated in Fig. 5.8. Preincubations with O3D1 and O4D2 yielded very similar results. This indicated that the sites recognised by I1A1, I4B1 and I4C3 on the Ogawa LPS were either identical to, or close to the binding sites of the Ogawa positive MCAbs.

5.11 Epitopes on V. cholerae LPS

The inhibition of binding of the apparent Inaba specific MCAbs to the Ogawa vibrios by O4A6, O3D1 and O4D2, demonstrated in Section 5.10. indicated that all antibodies shared epitopes which were closely related in spatial terms. However,this assay system had a very limited range over which competition could be

Fig.5.8 Detection of epitopes on LPS molecules by ELISA

Ogawa vibrios were preincubated with MCAb O4A6, washed and then reacted with other MCAbs. The bars represent a mean obtained from nine values.



Second Incubation with PBS

Second Incubation with O4A6

Second Incubation with O3D1 or O4D2

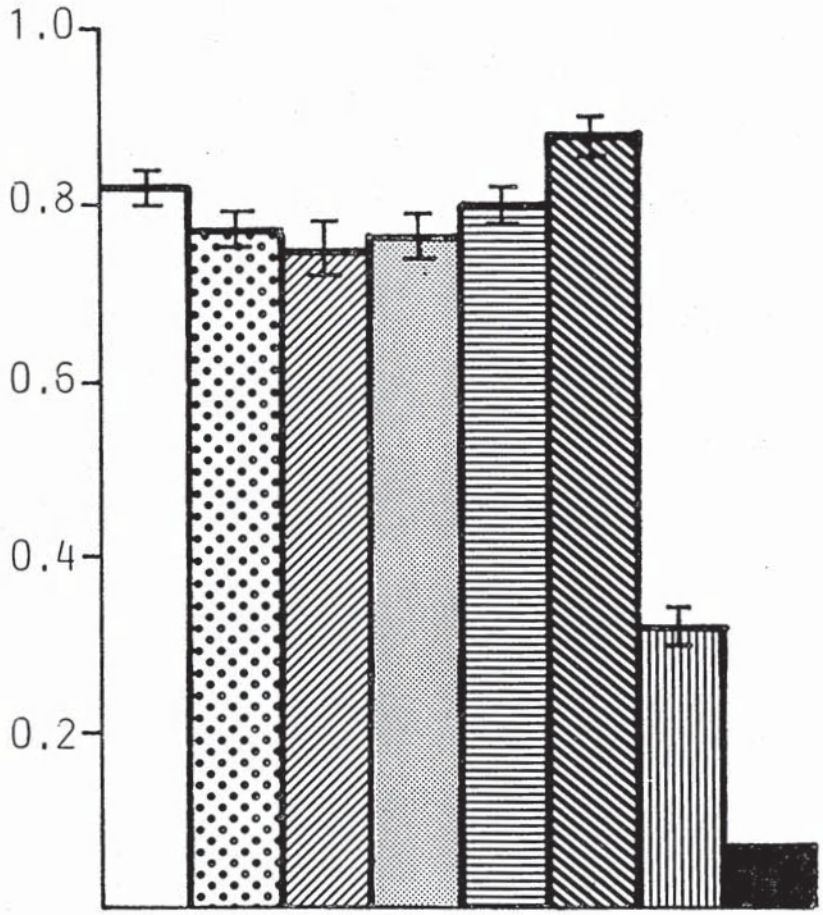
Second Incubation with I1A1, I4C3 or I4B1

Incubation with O3D1 alone

Incubation with O4D2 alone

Incubation with either I1A1, I4C3 or I4B1 alone

Background reaction


1.0
0.8
0.6
0.4
0.2

tested. In addition, previous assay systems of the ELISA type had given an innaccurate picture of fine specificity. Direct binding competition studies with purified antibodies can obviously yield less equivocal data over a much wider range and consequently the antibodies were purified from tissue culture supernatant.

5.12 Purification of Monoclonal Antibodies

Antibodies may be purified on affinity columns coated with suitable antigens or on columns coated with protein A (Goding, J.W. 1978). The rat MCAbs raised against the *V. cholerae* LPS were all of the IgG2b type which was reported to have a low affinity towards protein A of *S. aureus* (Rousseaux *et al*, 1981; Nilsson *et al*, 1982) and the preparation of antigen-coated affinity column was not considered to be feasible. Consequently the techniques employed for the purification of the antibodies generated in this work were those involving ion exchange columns.

5.12.1 Purification of rat IgG on DEAE Affi-gel blue

Affi-gel blue and DEAE Affi-gel blue have been reported to separate IgG from serum albumin. MCAbs from mouse ascites were purified by Bruck *et al*, (1982) on DEAE Affi-gel blue. The purification of the rat MCAbs was attempted on DEAE Affi-gel blue as described in Section 2.3.4.4.

The fractions that were collected from each column were estimated for protein by measuring the absorbance at 280nm and antibody activity on ELISA at 492nm. About 50% of the total

antibody activity co-eluted with albumin at a salt concentration of 1.4M (Fig. 5.9). Higher ionic strength was used to elute the non-Ig proteins from the column. The fractions containing the least amount of protein and maximum antibody activity were electrophoresed on 10% acrylamide-SDS gels and stained with coomassie blue. Every fraction showed contaminating albumin and no Ig bands were detectable. Very small amounts of tissue culture supernatant could be purified by this method and the net yield of Ig was not satisfactory for further use. This led to the investigation of other approaches for the purification of the antibodies from cell culture supernatant.

5.12.2 Purification of antibodies on a QAE sephadex column

QAE sephadex has been employed for the separation of rabbit IgG from serum albumin. At the pH the separation is conducted, pH 6.5, the IgGs have almost no net charge and hence are eluted in the void volume. For the purification of the MCAbs, 50ml of culture supernatant of each hybridoma were concentrated by the ammonium sulphate precipitation method to 1-1.5ml. The concentrated antibodies were then applied onto a QAE sephadex A50 column (Section 2.3.4.4.2) and the fractions collected. Each fraction was estimated for the protein content by absorption at 280nm and for anti-*V. cholerae* activity by ELISA on the appropriate serotype of vibrios by absorption at 492nm.

Two very distinct profiles of protein content and anti-*V. cholerae* were obtained from the fractions collected for different antibodies. Fig. 5.10a is the picture of I1A1 fractions

Fig.5.9 Purification of MCAbs on DEAE Affi-gel Blue

MCAb I1A1 was purified on DEAE Affi-gel Blue as described in Section 2.3.4.4.1.

×——× ELISA activity on Inaba vibrios

———— Protein content as measured by optical density at 280nm

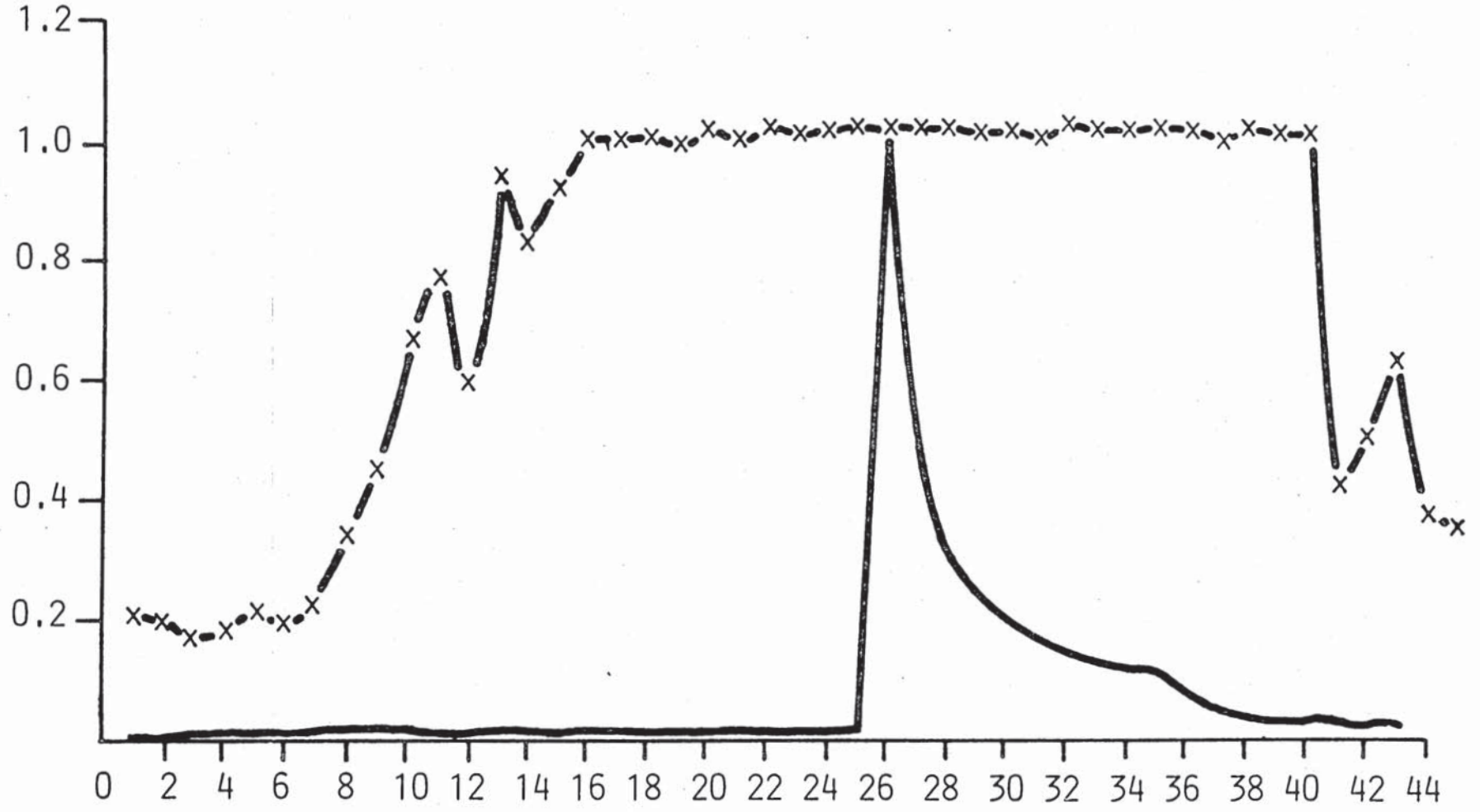
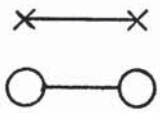

1.2
1.0
0.8
0.6
0.4
0.2
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44

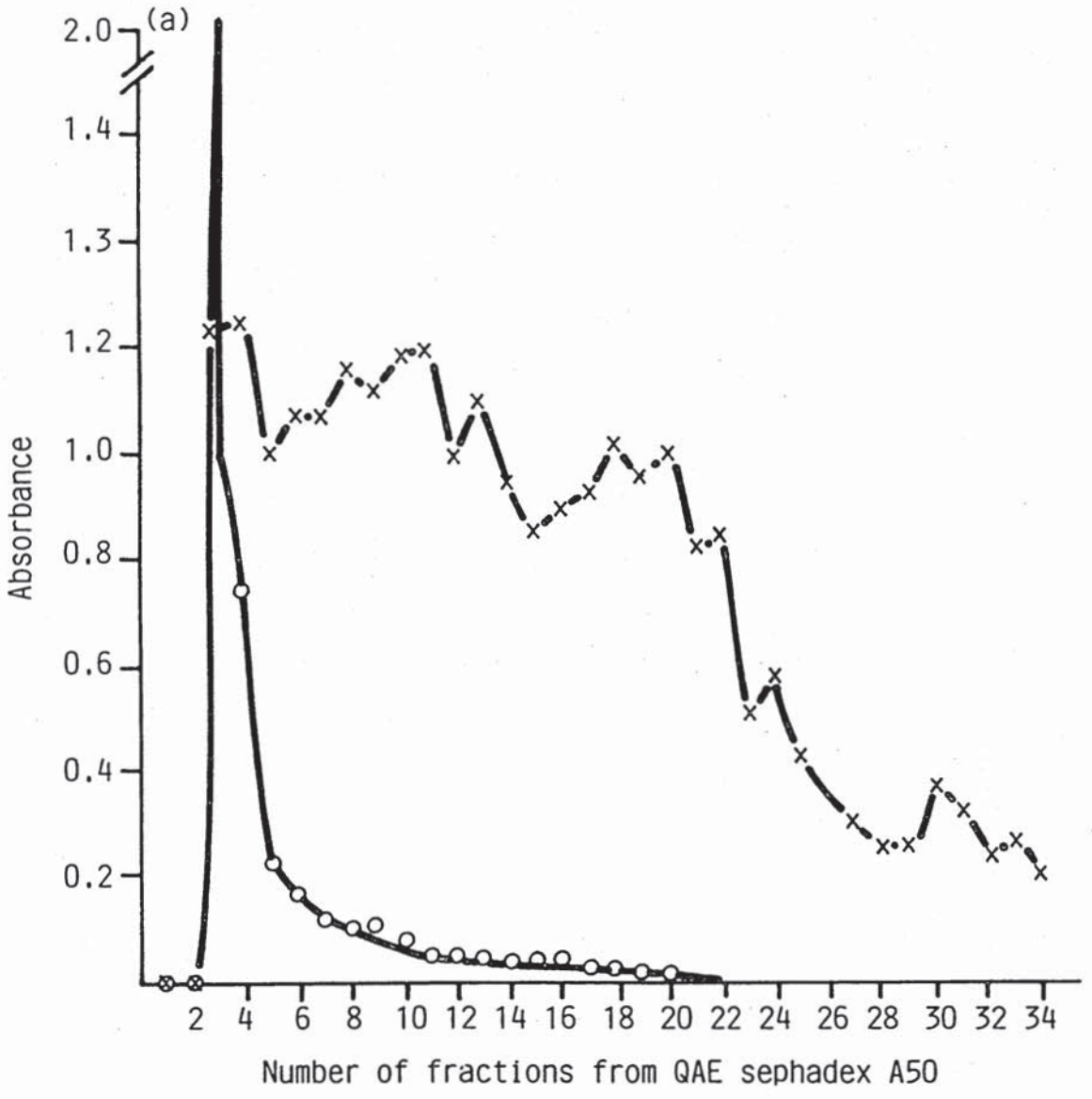
Fig. 5.10 a Purification of MCAb on QAE Sephadex A50 column

MCAb I1A1 was purified on QAE Sephadex column as described in Section 2.3.4.4.2.



ELISA activity on Inaba vibrios

Protein content as measured by optical density at 280nm


(a)
2.0
1.4
1.3
1.2
1.0
0.8
0.6
0.4
0.2
Absorbance
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
Number of fractions from QAE sephadex A50

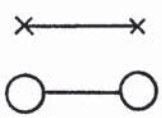
from the QAE column. I4C3 and I4B1 antibodies behaved in a very similar fashion. The protein content and the antibody activities of O3D1 have been illustrated in Fig. 5.10 b. O4A6 and O4D2 gave identical patterns when purified on the QAE sephadex column. Every antibody was purified at least twice and it was always observed that the Ogawa specific MCAbs yielded much lower amounts of immunoglubulins than the Inaba specific antibodies (Table 5.7). Since the Ogawa specific antibodies had good ELISA activity before purification, it is presumed that the Ogawa specific antibodies had a significantly higher affinity for the QAE sephadex and were less suited to purification by this method, which is one originally developed for the isolation of bulk IgG from polyclonal antisera. The method, however, yielded adequate amounts of of all six antibodies for further study.

5.12.3 Microheterogeneity in anti-Inaba MCAb molecules

The anti-Inaba MCAbs eluted over a large number of fractions and this was in contrast to the elution pattern obtained with anti-Ogawa MCAbs. The heterogeneity in the migration of the anti-Inaba antibodies on the column could have been due to partial binding by some population of the antibody molecules. If it is true, it suggests that the whole population of all three anti-Inaba MCAbs are not homogenous in their net charge.

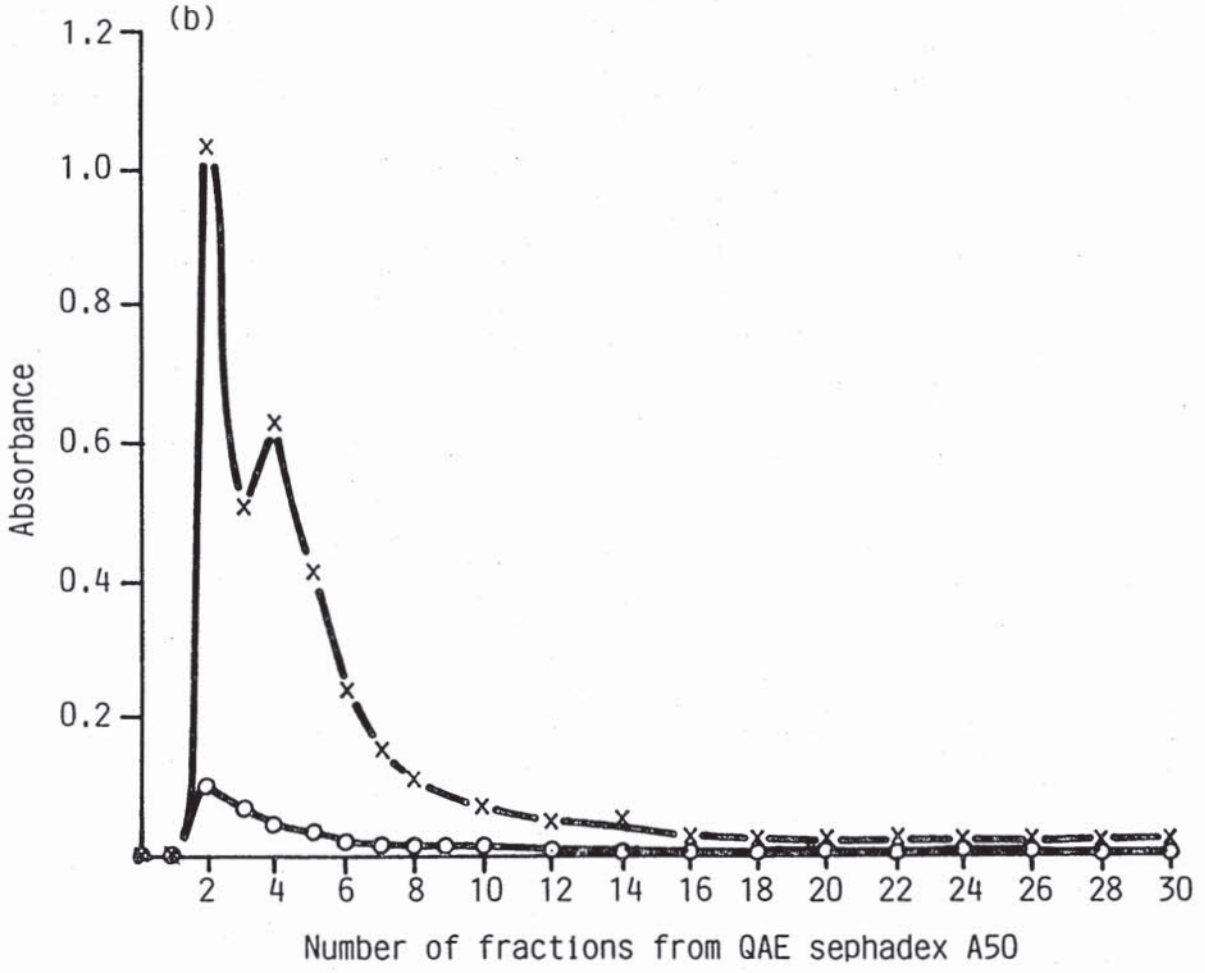
Fig. 5.10 b Purification of MCAb on QAE Sephadex A50 column

MCAb O3D1 was purified on QAE Sephadex column as described in Section 2.3.4.4.2.



ELISA activity on Ogawa vibrios

Protein content as measured by optical density at 280nm


(b)
1.2
1.0
0.8
0.6
0.4
0.2
Absorbance
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Number of fractions from QAE sephadex A50

Sample	Volume ml	A_{280}	Protein mg/ml	Total protein mg
I4B1(1/10)	2.5	0.33	0.23	5.75
O3D1	1.8	0.36	0.25	0.45
O4D2	1.3	0.27	0.19	0.25

Table. 5.7 Yield of Immunoglobulins on purification of MCAbs

MCAbs concentrated from culture supernatant by ammonium sulphate precipitation were purified on QAE-Sephadex column.

5.12.4 SDS-PAGE of purified immunoglobulins

The fractions collected from the QAE sephadex column of each antibody were pooled according to the activities exhibited on ELISA. The pooled fractions were lyophilised and then regenerated as described in (Section 2.3.4.4.2). The regenerated antibodies were electrophoresed to test the purity of the immunoglobulins. Fig. 5.11 illustrates the purity of O4D2 and I4B1 after being stained with coomassie blue. The purified samples of the other antibodies also contained no detectable traces of albumin.

5.13 Affinity tests of MCAbs at equivalent protein concentrations

The purified and regenerated samples of O4D2, O3D1 and I4B1 were estimated for protein by measuring the absorption at 280nm. The protein content of the three antibodies were adjusted to 0.19mg per ml and serial doubling dilutions were made starting with the stated concentration. Dilutions of the antibodies prepared were reacted with 10^6 Ogawa or Inaba vibrios on ELISA. This led to the observation that at the same concentration of immunoglobulins, I4B1 had maximum activity against the Inaba vibrios and this was followed by the activity of O4D2 and O3D1 against the Ogawa vibrios. Some binding of the I4B1 to Ogawa was also detected (Fig. 5.12). There was no binding of the O3D1 and O4D2 to the Inaba at the levels of antibody and antigen used.

Fig. 5.11 SDS-PAGE of purified MCAbs

MCAbs purified on QAE Sephadex column were analysed on SDS-PAGE for detection of contaminating proteins. The gel was stained with coomassie blue.

Lane 1 Protein molecular weight markers

Lane 2 Purified O4D2

Lane 3 Purified I4B1

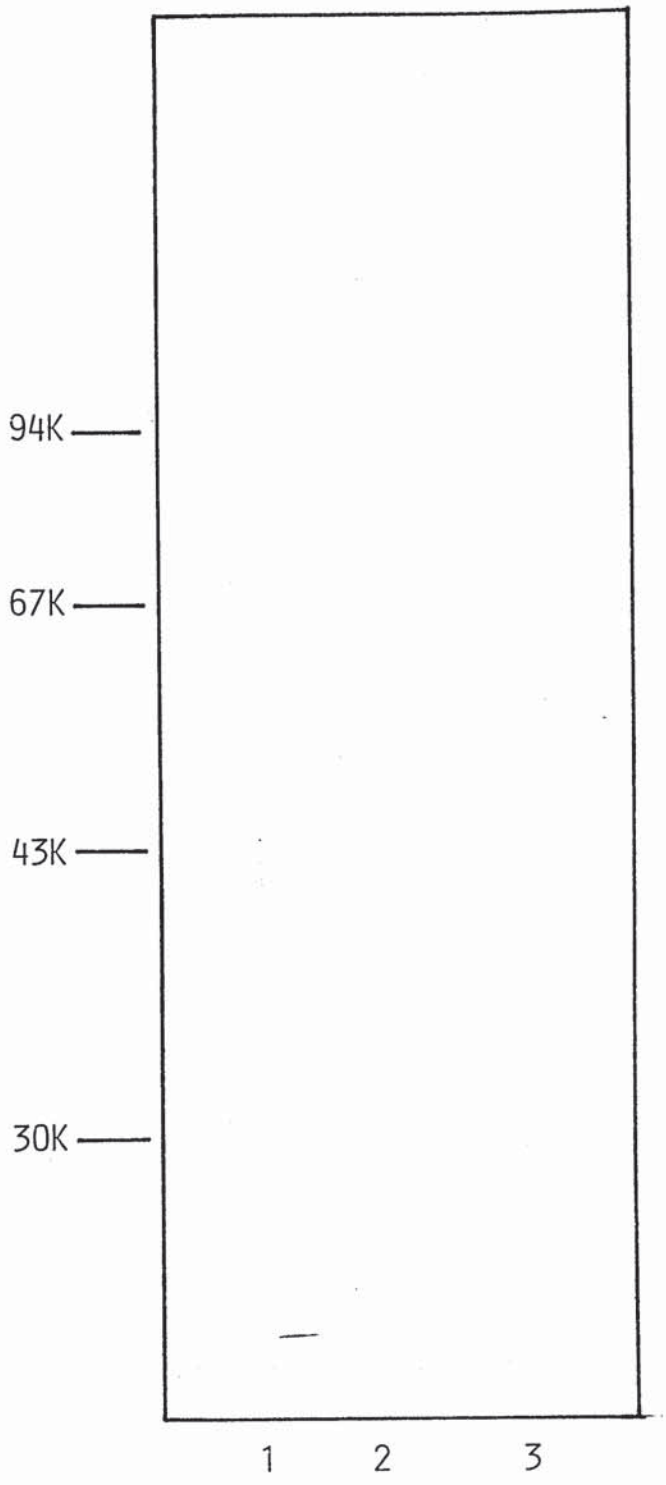
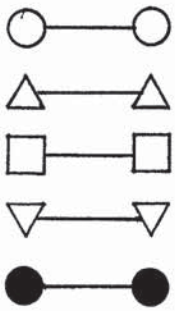

94K
67K
43K
30K
1
2
3

Fig. 5.12 ELISA of purified MCAbs

Purified MCAbs were adjusted to an identical protein concentration (0.19mg/ml) and titred on vibrios.



I4B1 reacted with Inaba

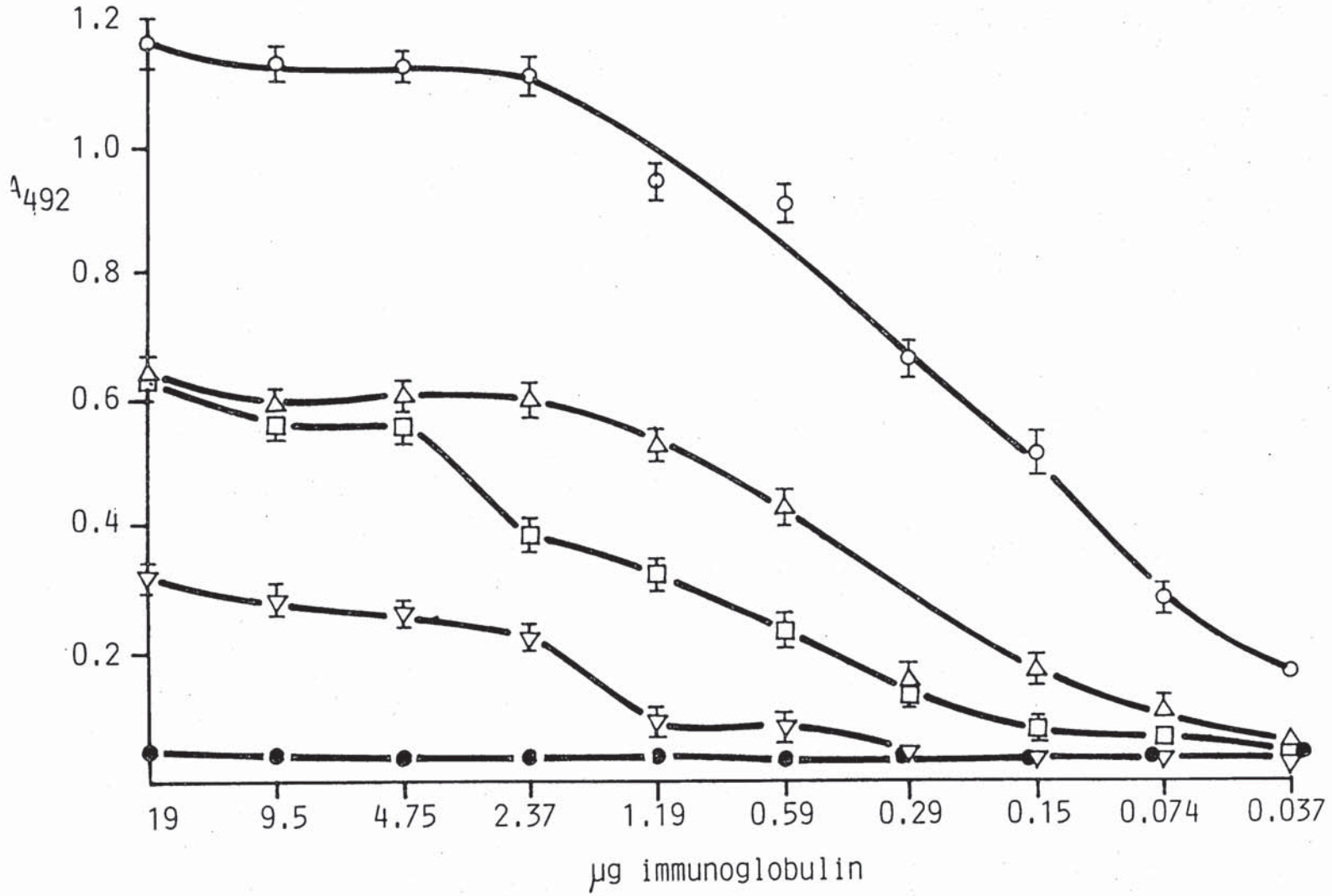
O3D1 reacted with Ogawa

O4D2 reacted with Ogawa

I4B1 reacted with Ogawa

Average of O3D1 and O4D2 reacted with Inaba

Background reaction was 0.05 O.D.


1.2
1.0
A_{492}
0.8
0.6
0.4
0.2
19
9.5
4.75
2.37
1.19
0.59
0.29
0.15
0.074
0.037
µg immunoglobulin

These data suggest that either I4B1 has higher affinity for Inaba vibrios than O3D1 and O4D2 for Ogawa vibrios or that there are more available epitopes on the Inaba organisms. However, it is also possible that some antibody activity was differentially lost during the purification procedures.

5.14 Radio-iodination of purified MCAbs

About 1mg of I1A1, I4B1 and I4C3 were iodinated with 500uCi of $Na^{125}I$ and 500ug of O4A6, O4D2 and O3D1 were iodinated with 300uCi of $Na^{125}I$. 2-8x 10^7 cpm of labelled antibodies were obtained by this procedure. The process of purification and iodination of the antibodies was repeated twice for each MCAb.

5.15 Radioactive binding assays with iodinated immunoglobulins

Direct binding and inhibition studies were carried out using the labelled antibodies. This analysis removed doubts about possible discrepancies introduced by the second antibody, and made it possible to confirm that the epitopes of the antibodies overlapped sufficiently to preclude simultaneous binding of two types of antibody at the same time.

5.15.1 Determination of vibrio concentration required for studies with labelled antibodies

Constant amounts of labelled antibodies were tested on increasing numbers of vibrios to detect the appropriate concentration of vibrios to be used for binding studies. It was particularly necessary to use high amounts of antigen for the

detection of the cross-reactivity of the anti-Inaba antibodies. The binding of I1A1 increased linearly up to 10^8 vibrios and then formed a plateau at higher concentrations of the vibrios (Fig. 5.13). This is in contrast to Figure 5.5c where a biphasic binding curve was observed in the ELISA assay utilising a second antibody, and suggests that second antibody effects were indeed responsible for this phenomenon. However, both the total antibody concentration and the type of plate employed were other contributory variables.

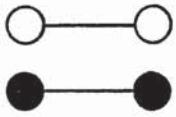
5.15.2 Direct binding of antibody to a variety of gram-negative bacteria

Linbro flexible plates were coated with a variety of bacteria at the concentration 10^9 organisms per well. The bacteria used for the test were the two serotypes of V. cholerae, B. abortus, B. melitensis and E. coli (ML 308). The labelled immunoglobulins were applied to each sample of bacteria and allowed to react as described in (Section 2.3.3.6) and the extent of the reaction of each MCAb with the various bacteria was measured. A direct comparison between each MCAb was not possible since the specific activity of the antibodies was not determined.

None of the six MCAbs produced in this study bound to B. abortus, B. melitensis or E. coli. O3D1, O4D2 and O4A6 did not bind to Inaba. An appreciable amount of I1A1, I4B1 and I4C3 bound to the Inaba vibrios (Table 5.8 a) and O4A6, O4D2 and O3D1 demonstrated high binding to the Ogawa vibrios. Cross reactive binding of I1A1, I4B1 and I4C3 to Ogawa was significant but the

Fig.5.13 Reaction of labelled antibody on increased antigen density

$4x10^6$ cpm of labelled I1A1 was reacted with different antigen concentrations. The values plotted are a mean of two assays.



cpm bound on Ogawa vibrios

cpm bound on Inaba vibrios

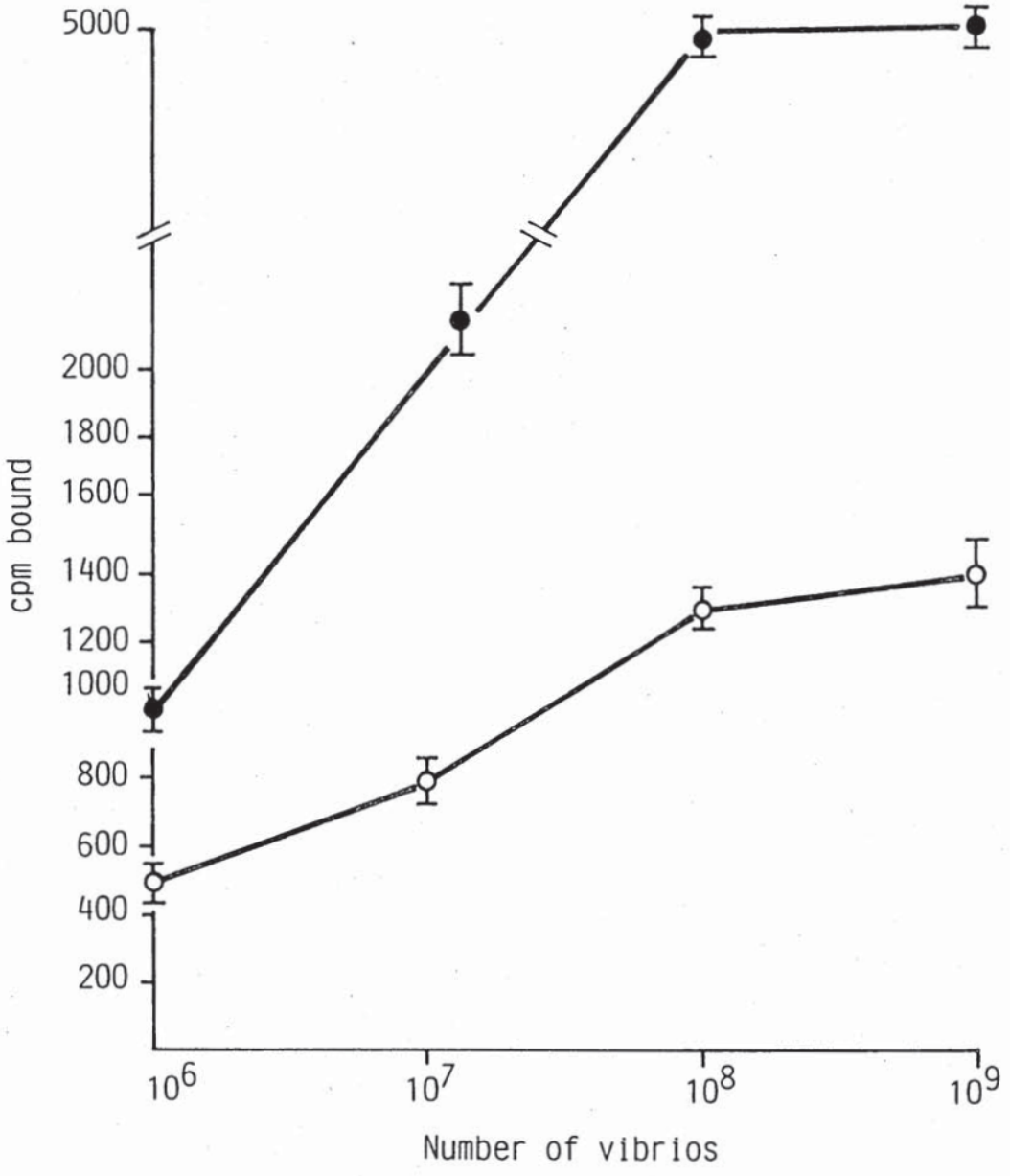

5000
2000
1800
1600
1400
1200
1000
800
600
400
200
cpm bound
10^6
10^7
10^8
10^9
Number of vibrios

Table 5.8 a Binding studies of radiolabelled MCAbs to various gram-negative bacteria

Each radiolabelled antibody was applied to various organisms and the amount of antibody bound to the bacteria was determined as described in Section 2.3.3.6.

B.A B.abortus

B.M B.melitensis

E.C E.coli

MCAb	cpm applied	No. of assays	Bk.	c p m ± S.D.				
				Inaba	Ogawa	B.A.	B.M.	E.C
IA1	$2\text{-}5\times10^6$	6	100	7500 ±250	1200 ±85	140 ±13	130 ±16	130 ±10
I4C3	$1\text{-}2\times10^6$	4	60	6200 ±500	510 ±25	50 ±5	70 ±10	70 ±12
I4B1	$1\text{-}4\times10^6$	4	100	4700 ±335	1110 ±55	135 ±16	110 ±9	200 ±15
O4D2	$5\text{-}8\times10^6$	4	50	200 ±95	3090 ±250	80 ±10	120 ±14	350 ±10
O3D1	$1\text{-}2\times10^6$	4	60	290 ±19	5670 ±215	70 ±5	200 ±15	300 ±17
O4A6	$2\text{-}3\times10^6$	2	75	315 ±25	4800 ±300	95 ±15	115 ±9	210 ±10

cross reaction of the three Ogawa antibodies,including O3D1, with Inaba was very weak.

5.15.3 Inhibition assays with radiolabelled antibody

The binding sites of the labelled antibodies to Ogawa or Inaba vibrios were inhibited by cold antibodies to investigate two possibilities. Firstly, it was necessary to determine whether the apparently specific epitopes on the two vibrios were independent or whether the two basic types of monoclonal antibodies could block each other. Secondly, it was of interest to determine whether cross reactions between serotypes could be abolished by the use of antibodies to the homologous serotype and whether cross reacting activity was distinct from homologous reaction. The data (Table 5.8 b, c, d and e) clearly indicated that I1A1, I4B1 and I4C3 had binding sites distinct from the O panel of antibodies on their own serotype but could be dislodged by the anti Ogawa antibodies when cross reacting with this heterologous serotype. O4A6, O3D1, and O4D2 were totally specific on their own serotype and unaffected by the presence of the anti-Inaba monoclonals. O3D1 showed no tendency to diverge from the other two members of the original O specific panel as it had on other assays such as agglutination or complement fixation.

5.16 Summary of assay results for the six monoclonal antibodies

5.16.1 MCAbs I1A1,I4B1 and I4C3

These antibodies reacted in a similar manner in all assays. Their relative reaction to the two serotypes is

Table 5.8 b Inhibition assay on Inaba vibrios

The vibrios were incubated with unlabelled MCAbs followed by labelled antibodies as described in Section 2.3.3.7.

\# Average cpm bound to Inaba vibrios

* Average cpm bound to Inaba vibrios after blocking with unlabelled MCAbs

MCAb	cpm appld	No. of assays	cpm[#] ±S.D. on Inaba	Inhibition by MCAbs to Inaba					
				cpm[*] ±S.D.			percentage		
				I1A1	I4B1	I4C3	I1A1	I4B1	I4C3
I1A1	$3x10^6$	6	7500 ±250	2100 ±100	2660 ±200	2250 ±95	72	65	70
I4B1	$2x10^6$	4	4700 ±335	2805 ±155	2919 ±200	2750 ±350	40	38	42
I4C3	$2x10^6$	4	6200 ±500	2860 ±400	3100 ±155	3215 ±205	54	49	48

Table 5.8 c Inhibition assay on Inaba vibrios

The vibrios were incubated with unlabelled MCAbs followed by labelled antibodies as described in Section 2.3.3.7.

Average cpm bound to Inaba vibrios

* Average cpm bound to Inaba vibrios after blocking with unlabelled MCAbs

\- No inhibition observed

MCAb	cpm appld	No. of assays	cpm[#] ±S.D. on Inaba	Inhibition by MCAbs to Inaba					
				cpm[*] ± S.D.			percentage		
				O4A6	O3D1	O4D2	O4A6	O3D1	O4D2
I1A1	$3x10^6$	4	7500 ±250	7800 ±100	7120 ±125	7700 ±200	-	-	-
I4B1	$2x10^6$	4	4700 ±335	5700 ±400	4900 ±150	4500 ±260	-	-	-
I4C3	$2x10^6$	4	6200 ±500	6100 ±100	6555 ±90	7005 ±115	-	-	-

Table 5.8 d Inhibition assay on Ogawa vibrios

The vibrios were incubated with unlabelled MCAbs followed by labelled antibodies as described in Section 2.3.3.7.

\# Average cpm bound to Ogawa vibrios

* Average cpm bound to Ogawa vibrios after blocking with unlabelled MCAbs

\- No inhibition observed

nd not determined

MCAb	cpm appld	No..of assays	cpm[#] ±S.D. Ogawa	Inhibition by MCAbs to Ogawa					
				cpm[*] ± S.D.			percentage		
				I1A1	I4B1	I4C3	I1A1	I4B1	I4C3
I1A1	$3x10^6$	6	1200 ±85	660 ±15	775 ±20	820 ±25	45	35	32
I4B1	$2x10^6$	4	1110 ±55	nd	nd	nd	nd	nd	nd
I4C3	$2x10^6$	4	510 ±25	455 ±20	340 ±35	290 ±20	10	33	43
O4A6	$2x10^6$	2	4800 ±300	4920 ±125	5125 ±300	4795 ±250	-	-	-
O3D1	$2x10^6$	4	5670 ±215	6600 ±500	7230 ±215	7290 ±310	-	-	-
O4D2	$6x10^6$	4	3090 ±250	3140 ± 30	3200 ± 27	3300 ± 85	-	-	-

Table 5.8 e Inhibition assay on Ogawa vibrios

The vibrios were incubated with unlabelled MCAbs followed by labelled antibodies as described in Section 2.3.3.7.

\# Average cpm bound to Ogawa vibrios

* Average cpm bound to Ogawa vibrios after blocking with unlabelled MCAbs

MCAb	cpm appld	No. of assays	cpm[#] ±S.D. Ogawa	Inhibition by MCAbs to Ogawa					
				cpm[*] ± S.D.			percentage		
				O4A6	O3D1	O4D2	O4A6	O3D1	O4D2
I1A1	$3x10^6$	6	1200 ±85	300 ±10	316 ±11	320 ±15	75	74	73
I4B1	$2x10^6$	4	1110 ±55	330 ±15	400 ±21	500 ±17	70	64	55
I4C3	$2x10^6$	4	510 ±25	170 ±15	160 ±16	120 ±19	67	69	92
O4A6	$2x10^6$	2	4800 ±300	3665 ±95	4110 ±105	3700 ±190	24	14	23
O3D1	$2x10^6$	4	5670 ±215	4600 ±100	4000 ± 95	4275 ± 65	19	29	25
O4D2	6x10	4	3090 ±100	1145 ±12	900 ±56	2010 ±145	63	71	35

summarised in Table 5.9 a. Thus, in general, the three "Inaba specific" MCAbs all showed a significant cross reaction with Ogawa vibrios and a weak but significant cross reaction with B. abortus

5.16.2 MCAbs 04A6 and 04D2

These MCAbs appeared to be totally specific on all assays. They did not agglutinate even homologous bacteria. They did, however, dislodge the cross reacting anti-Inaba monoclonal antibodies from their interaction, indicating that there was a close relationship between the epitopes of the two serotypes.

5.16.3 MCAb 03D1

03D1 behaved identically to the other 04A6 and 04D2 in all assays except two. In the agglutination assay, it weakly but persistently agglutinated Inaba (though not Ogawa) bacteria. In the complement fixation assay, it fixed complement in the presence of Inaba vibrios at a concentration 5-10 fold higher than that in which it gave activity with the homologous serotype. Such results are suggestive of a weak interaction with Inaba vibrios, significantly weaker than the interaction observed with the "Inaba specific" monoclonal antibodies with Ogawa vibrios.

Table 5.9 a Summary chart of MCAbs raised against Inaba

N.D. not determined

N.S. not significant

ASSAYS CONDUCTED	MONOCLONAL ANTIBODIES		
	I1A1	I4B1	I4C3
1. Titres of antibodies on ELISA			
(Section 3.6)			
Ogawa	no react.	no react.	no react.
Inaba	1 in 256	1 in 512	1 in 512
B.abortus	no react.	no react.	no react.
B.melitensis	no react.	no react.	no react.
E.coli	no react.	no react.	no react.
2. Reaction on immunoblotting			
(Section 4.17)			
Ogawa	no react.	no react.	no react.
Inaba	positive	positive	positive
B.abortus	no react.	no react.	no react.
E.coli	no react.	no react.	no react.
S.typhimurium	no react.	no react.	no react.
3. Competition ELISA			
(Section 5.1)			
50% inhibition with Ogawa LPS	no inhib.	no inhib.	no inhib.
50% inhibition with Inaba LPS	56ng	60ng	45ng
4. Quantitation of IF			
(Section 5.2)			
Ogawa	6	5.6	4.5
Inaba	14	14	9.7
Inaba / Ogawa preference	2.3	2.5	2.1

	I1A1	I4B1	I4C3
5. Slide Agglutination (Section 5.4.1)			
Ogawa	+	+	+
Inaba	+	+	+
B.abortus	+	+	+
B.melitensis	-	-	-
Y.enterocolitica	-	-	-
6. Antibody titre on primary agglutination (Section 5.7)			
Ogawa	2.5	1.25	1.25
Inaba	1.25	0.6	0.3
Inaba/Ogawa preference	2	2	4
7. Antibody titre on secondary agglutination(Section 5.4.2.2)			
Ogawa	1.25	1.25	1.25
Inaba	0.3	0.3	0.3
Inaba/Ogawa preference	4	4	4
8. Concentration of antibodies that fixed 50% complement (Section 5.7)			
Ogawa	0.034	12.31	0.044
Inaba	0.009	3.19	0.014
B.abortus	N.D	N.D	0.8
Inaba/Ogawa preference	3.7	3.8	3.2
Inaba/Brucella preference	N.D	N.D	57
Ogawa/brucella preference	N.D	N.D	18

	I1A1	I4B1	I4C3
9. Maximum activity on ELISA at 10^7-10^8 bacteria (Section 5.5.2)			
Ogawa	0.9	0.91	0.64
Inaba	2	2	1.68
B.abortus	N.S	N.S	N.S
B.melitensis	N.S	N.S	N.S
E.coli	N.S	N.S	N.S
Inaba/Ogawa preference	2.2	2.19	2.63
10. Direct binding assay (cpm) (Section 5.14.2)			
Ogawa	1200	1110	510
Inaba	7500	4700	6200
E.coli	N.S	N.S	N.S
B.abortus	N.S	N.S	N.S
B.melitensis	N.S	N.S	N.S

Table 5.9 b Summary chart of MCAbs raised against Ogawa

N.S. not significant

ASSAYS CONDUCTED		MONOCLONAL ANTIBODIES		
		O4A6	O4D2	O3D1
1. Titres of antibodies on ELISA				
(Section 3.6)				
	Ogawa	lin 512	1 in 512	1 in 512
	Inaba	no react.	no react.	no react.
	B.abortus	no react.	no react.	no react.
	B.melitensis	no react.	no react.	no react.
	E.coli	no react.	no react.	no react.
2. Reaction on immunoblotting				
(Section 4.17)				
	Ogawa	positive	positive	positive
	Inaba	no react.	no react.	no react.
	B.abortus	no react.	no react.	no react.
	E.coli	no react.	no react.	no react.
	S.typhimurium	no react.	no react.	no react.
3. Competition ELISA				
(Section 5.1)				
50% inhibition with Ogawa LPS		25ng	62.5ng	65ng
50% inhibition with Inaba LPS		no inhib.	no inhib.	no inhib.
4. Quantitation of IF				
(Section 5.2)				
	Ogawa	38	37	35
	Inaba	no react.	no react.	no react.

		O4A6	O3D1	O4D2
5.	Slide Agglutination (Section 5.4.1)			
	Ogawa	-	-	-
	Inaba	-	-	+
	B.abortus	-	-	-
	B.abortus	-	-	-
	Y.enterocolitica	-	-	-
6.	Antibody titre on primary agglutination (Section 5.4.2.1)			
	Ogawa	5	20	5
	Inaba	-	-	2.5
	Ogawa/Inaba preference	-	-	1/2
7.	Antibody titre on secondary agglutination (Section 5.4.2.2)			
	Ogawa	0.6	20	2.5
	Inaba	10	20	2.5
	Ogawa/Inaba preference	16	1	1
8.	Concentration of antibodies that fixed 50% complement (Section 5.7)			
	Ogawa	v.dil.	0.19	0.0038
	Inaba	-	-	-
	Ogawa/Inaba preferrence	-	-	79

		O4A6	O4D2	O3D1.
9. Maximum activity on ELISA at 10^7-10^8 bacteria (Section 5.5.2)	Ogawa	1.8	2	1.65
	Inaba	N.S.	N.S.	0.65
	B.abortus	N.S.	N.S.	N.S.
	B.melitensis	N.S.	N.S.	N.S.
	E.coli	N.S.	N.S.	N.S.
Ogawa/ Inaba preference		-	-	2.5
10. Direct binding assay (cpm) (Section 5.14.2)	Ogawa	4800	3090	5670
	Inaba	N.S.	N.S.	N.S.
	E.coli	N.S.	N.S.	N.S.
	B.abortus	N.S.	N.S.	N.S.
	B.melitensis	N.S.	N.S.	N.S.

GENERAL DISCUSSION

6.1 The immune response to V.cholerae

Clinical infections of V. cholerae give rise to both antibacterial and anti-toxin antibodies (Majumdar and Ghose, 1982; Holmgren and Svennerholm, 1973), although antibacterial antibodies have been found to be more effective in vibriocidal activity (Levine et al, 1979). Cryz et al, (1982) have shown that absorption with LPS of antisera from animals immunised with viable vibrios leads to a decrease in the vibriocidal activity. They estimated that approximately 75% of the vibriocidal activity was directed against the LPS and the rest against non-LPS surface antigens. The type specific antigens of V. cholerae have been serologically defined by absorption studies (Kauffmann, 1950; Gardner and Venkatraman, 1935) and Gustafsson and Holme (1983) have produced MCAbs with specificities conforming to the classically defined A, B and C antigens. However, no immunological data relating to the structural inter-relationships among these antigens has yet been reported. In this study, the fine specificity of the MCAbs raised against the LPS of Inaba and

Ogawa serotypes has been used to investigate the stuctural homologies among the epitopes on the complex LPS molecules of the two serotypes.

6.2 Factors leading to the preferential selection of MCAbs reactive with LPS

The fact that the six monoclonal antibodies in this study were all reactive with the bacterial LPS may be chance or due to the immunisation and selection procedures. The animals used for the production of the MCAbs were immunised with heat killed whole cells of either Ogawa or Inaba serotype (Section 2.3.1.1). As the LPS represents several percent of the dry weight of gram-negative bacteria and accounts for many of their antigenic properties, a strong response against the V. cholerae LPS would therefore be anticipated. However, immunisation with cells could have also led to the production of antibody directed against membrane proteins of the bacteria. Kabir (1980) and Stevenson et al, (1985) have reported the presence of immunogenic proteins in the outer membrane of V. cholerae. Outer membrane proteins have been used by other workers (Sciortiono et al, 1985) for raising MCAbs to V. cholerae membrane proteins. Anti-protein activities of vibriocidal antibodies have also been demonstrated by Neoh and Rowley (1970; 1971). In this study, rat anti-V. cholerae antiserum reacted with Western blots of cell sonicates showing staining of a wide range of proteins (data not shown). The MCAbs established from the immunised rats, however, reacted with only the LPS component of the bacteria.

6.2.1 Final boosting of experimental animals

The final boost, 4 days prior to fusion, is generally agreed to be vital to the production of a large number of MCAbs (Stahli et al, 1980). It is possible to suggest that the LPS molecules on the outer surface of the bacteria dominate this procedure as they are effectively seen first by the immune system, with the other antigens only being able to elicit a response after the bacterium has been further degraded, possibly by antigen presenting cells with the subsequent involvement of T lymphocyte help (see Section 6.2.3).

6.2.2 Selection of hybridomas by assay

V. cholerae cells were used in this study for the screening of hybridoma supernatants from emerging clones. As LPS is localised almost exclusively on the outer surface of the outer membrane it would have been readily accessible at high density to the antibodies from the emerging hybdridomas during selection. The membrane proteins on the other hand may have been less accessible for interaction with the antibodies. Thus either accessibility, or sheer epitope density, may have favoured MCAbs to LPS at this stage with the clones giving a lower signal for membrane proteins being rejected as only weak positives.

6.2.3 The polyclonal effect of bacterial LPS on B-cells

Bacterial cell wall LPS is well known for its ability to induce a Thymus Independent (TI) polyclonal activation of

B-cells(Andersson et al, 1972) in addition to many striking pathophysiological effects on mammalian organisms (Morrison and Ryan, 1970). Although the exact mechanism of activation of LPS on the proliferation and maturation of B-cells is not defined, considerable progress has been made in sorting out the multiple effects of LPS on B lymphocytes (Section 1.16). The correlation between antigenic activity of the three originally classified anti-Inaba antibodies with other bacteria expressing mannopyranosyl sugars is of particular interest and may suggest that these play a significant role as adjuvants, comparable with that of the mannose associated O chains (Section 1.16).

6.2.4 Specific effects of V.cholerae LPS as antigen

In this study V. cholerae organisms have been used as immunogens and have resulted in antibodies against LPS. The Lipid A moiety of E. coli LPS can induce B-cells to proliferate and mature into antibody producing cells (Raetz et al, 1983) and it may therefore have been possible for Lipid A alone to have induced the B-cells into producing increased amounts of antibodies against the immunodominant O-region.

6.3. Antigenic components of the core LPS

The inner core region of the LPS is considered to be of great biomedical importance since it contains immunogenic structures that are common to many pathogenic gram-negative bacteria (Luderitz et, al, 1966) (Section 1.14).

The MCAbs established in this study do not react strongly

with any of the core components of V. cholerae LPS as seen in the Western blots (Section 4.8). This does not exclude the possibility of the existence of weak interactions of the antibodies with the core which were not detected in the Western blots because of the limited sensitivity of the method in comparison to the two non-washing methods employed. Some of the cross-reactions observed between the Inaba and the Ogawa serotypes and between Inaba and B. abortus may be due to the recognition of the core components. However, the core portions of the LPS of most gram-negative bacteria are thought from conventional serology, to be very similar and to share antigenic determinants. Consequently, the cross-reactive anti-Inaba MCAbs or the O3D1 anti-Ogawa antibody would have been expected to react to some extent with more of the other gram-negative bacteria tested. Thus the cross-reactive MCAbs appeared to detect only similarities between the O-antigens of the different bacteria tested. Steric factors in both parts of the LPS may also affect the access of a single monoclonal antibody where they do not significantly affect the reaction of polyclonal antiserum.

Immunisation and selection protocols may have played a part in the stimulation and detection of the antibodies to O regions in the same way as they may have influenced the selection of LPS as major antigen (Section 6.2). The majority of anti-core antibodies have been obtained either by immunisation with mutant rough type organisms or by selection of the hybridomas against the purified LPS of the immunising organism. In both cases, the core may be better exposed in vivo to the

immune system or *in vitro* to the selection system. The latter possibility is particularly logical since it seems likely that the core is more accessible to the antibodies in the purified form of the LPS rather than on the complex bacterial surface where it may remain embedded under the immuno-dominant O-side chains. This conclusion is supported by the results of Perez *et al*, (1985) who from their studies with antisera raised against rough, intermediate and smooth types of *Campylobacter* have proposed two hypotheses. Firstly, the core structure on smooth type organisms is hidden by the O-polysaccharides so that the immune system does not see it. Secondly, the lack of response to core antigens of smooth-type LPS may be caused by the immunodominant O-determinants that may preempt the entire immune response. The authors are in favour of the first hypothesis because they were able to produce anti-core and anti-O antibodies from immunising rabbits with intermediate type organisms. However, such an interpretation is in contrast to the observations of Nelles and Niswander (1984) who observed that the anti-core antibodies do not bind to LPS derived from the parental smooth-type bacteria but are able to bind to the bacteria itself.

6.4 Analysis of the IgG subclass of the MCAbs

All six of the rat MCAbs against *V. cholerae* LPS generated in this work were of the IgG2b type. Statistically, since this is a minor serum subclass, such a grouping is unlikely. Mouse MCAbs established by others against bacterial LPS

have been predominantly of the isotypes IgM or IgG3 (Quinn et al, 1984; Gustafsson and Holme, 1983; Lind et al, 1985). This suggests a preferential LPS or carbohydrate association of the IgG3 isotype of MCAbs with antigens of this chemical constitution in the mouse. The rat equivalent, by analogy, would appear to be IgG2b. Isotype switching is not readily observed by cloned cells in culture but bulk culture of uncloned murine B lymphocytes responding to secondary LPS has been shown to generate additional IgG3 production from cultures previously producing IgM alone. (Jones et al, 1983; Vitetta et al, 1984; Bjorklund and Coutinho, 1983). Similar evidence has also shown that additional T-cell-derived factors are necessary for the expression of isotypes other than IgG3.

The heavy chain locus for IgG3 in mouse DNA is the one nearest to the delta chain coding region, albeit 55Kb downstream (Marcu, 1982; Shimizu and Honjo, 1984). It will be of interest to note, when the rat heavy chain genes are sequenced, if rat IgG2b genomic coding sequences occupy a similar and comparatively close position or whether the location is incidental.

6.5 Effect of phenol on LPS preparations

Aqueous phenol has been used for many years as a general method for the isolation of LPS from bacterial cells (Westphal and Jann, 1965). The deproteinizing action of hot aqueous phenol is known to cause cleavage at the point of attachment of lipid A to the protein moiety on the membrane. However, Tsang et al,

(1974)have reported that that the action of phenol is at a phenol-sensitive linkage within the lipid A moiety. Hydrolysis of the O-specific chain and fatty acids of the core were also reported to occur but only in negligible amounts.

V. cholerae LPS used for this study was extracted by hot aqueous phenol from the vibrios several times and no difference between the preparations was observed on SDS-PAGE analysis. The unextracted LPS in the sonicated vibrios and the crude LPS obtained by hot aqueous phenol extraction exhibited identical mobilities, and hence the hot aqueous phenol was not considered to have affected the O-antigen or the lipid A core complex significantly during the process of extraction.

6.6 Analysis of V.cholerae LPS on SDS-PAGE

6.6.1 O-side chain microheterogeneity in V.cholerae LPS

V. cholerae LPS has much greater size homogeneity than other LPS molecules previously analysed such as those from E. coli and S. typhymurium (Palva and Makela, 1980; Goldman and Leive, 1980). In this work the apparently homogenous LPS of V. cholerae has been shown to possess microheterogeneity which could be resolved only after high resolution electrophoresis. The complete O-chain of the LPS migrated as a single but diffuse band when electrophoresed under normal conditions. The existence of more than ten bands in the O-region was demonstrated on final resolution of the LPS (Section 4.14.2 and 4.14.3). It was, however, noticed that a background stain interspersed with the O-chain bands. This could mean that there were some glycans in

the LPS which did not migrate as sharp bands but formed a continuous smear.

V. cholerae LPS is a homopolysaccharide composed of perosaminyl residues, the amino groups of which are acylated by 3-deoxy-glycero-tetronic acid (Section 1.13.2). Very small differences in the mobilities of the bands forming the ladder suggest that the increments between them are also very small. The most obvious addition to each of the lower bands would be that of a perosamine residue. The repeat unit for the O-chain of the V. cholerae LPS would thus consist of only perosamine residues. The number of residues forming a repeat unit has not yet been defined. The mean chain length of the O-chain has also not been reported. The close spacing between the bands, however, indicates that the repeat unit is likely to be one perosamine residue only. An attempt to verify the suggestion was made by estimating the molecular weights of the O-chains from a calibration graph of mobility versus molecular weight of LPS molecules with known molecular structures, such as of E. coli 0111 B4 and S. typhimurium. The results indicated that the O-region of the V. cholerae consisting of more than ten bands was concentrated within M_r of 1400. In such a case the difference between each band would be about 140 which falls short of the molecular weight of an acylated perosamine residue which is 247. However, this method of molecular weight estimation, which is routinely used for proteins with reference to protein standards, may have limited applicability to LPS molecules both for the determination of the overall molecular weight of the core plus O antigen and

the molecular weight increment between two molecules with different numbers of O antigens. Where most proteins bind SDS in a uniform manner, the association of SDS with LPS involves only the hydrophobic lipid A moiety (Jann et al, 1975). The diversity that exists in the O-region of gram-negative bacterial LPS molecules may give rise to unique migration patterns on gel electrophoresis because of the variation in charge and chemical composition of the sugar residues. Even with proteins, which do bind SDS, mobility in gels can be shown to depend on conformational factors and also charge so that for example, the highly charged histones migrate abnormally slowly, as do glycoproteins (Hames and Rickwood, 1981). Consequently, the use of LPS of different charge and chemical composition as a standard for the analysis of LPS from another source has limited validity.

Redmond's (1979) observation of a molecular weight of about 10,000 for 60 perosamine residues is consistent with the small band difference obtained here.

An unequal distribution of the repeat units has been observed for E. coli 0111 B4, S. typhimurium and Citrobacter strain 396 LPS (Palva and Makela, 1980; Goldman and Leive, 1980). Each organism showed a preferred range of repeat units for the expression of the O-side chains. In contrast, the V. cholerae LPS extracted from the Ogawa NIH 41 and Inaba 35 A3 serotypes did not exhibit such wide variation in the O-side chain lengths, though the longer chains were in most abundance as seen in Figure 4.9. The presence of more than one diffuse band of O-antigen in the LPS of Inaba (569 B) could be a characteristic

of that strain or it may be a result of aggregation of the low molecular weight bands (Section 6.7.1). Since the length of O-side chain most abundantly expressed by Inaba 569 B coincided with the lengths expressed by Ogawa NIH 41 and Inaba 35 A3 it may be that this particular length of side chain is the range preferred by the V. cholerae organisms.

The increment in molecular weight between the bands was observed to be the same for Ogawa and Inaba serotypes. This is interesting because only the Ogawa polysaccharides has been reported to contain the sugar 4-amino-deoxy-arabinose (Redmond, 1978) and its position in the LPS has not been identified.

6.6.2 Mobilities of the core and the lipid A

The lipid A and the core of the V. cholerae LPS had identical mobilities to that of the E. coli 0127 B8 (Figure.4.7) and this is consistent with the findings that the core-lipid A complexes of gram-negative bacterial LPS are conserved. However, the doublet of the core-lipid A obtained in short gels (Figure. 4.2 a and b) was resolved into three bands with high resolution electrophoresis (Figure. 4.9). The topmost band of the three, which was stained very faintly could either indicate the presence of a complete core with the addition of one perosamine residue or heterogeneity in the lipid A region as reported by Rosner et al, (1979) in E. coli K12 LPS. The heterogeneity in the lipid A was reported by these authors to be caused by differences in phosphate groups which introduce a change in charge and hence a slight alteration in the mobility.

6.6.3 Sensitivity of silver stain for identification of LPS

The small quantities of V. cholerae LPS used for analysis on SDS-PAGE by silver staining is not inconsistent with the lower limit of carbohydrate content required for this detection system as estimated by Dubray and Bezard, (1982). It must be noted that Tsai and Frasch (1982) also reported their inability to stain free lipid A as was experienced in this study (Section 4.18 and 4.19).

6.7 Aggregation of LPS

6.7.1 Aggregation of LPS during electrophoresis

Migration of LPS has been shown to be affected by the formation of multimers in SDS-PAGE by Peterson and McGroarty (1985). The authors found that the banding patterns of LPS in SDS-PAGE were influenced by both the LPS and SDS concentrations used. Nanogram quantities of E. coli LPS were reported to form noncovalently associated multimers in gels run in 0.1% SDS. The multimers migrated more slowly than their monomeric substituents, causing anamolous bands either to appear in between, or to overlap with thus darken, longer O-antigen monomer bands. The multimers were moderately stable since they migrated as distinct bands and not as smears. Hitchcock (1983) has also described the dissociation of slow-migrating bands into faster migrating ones as well as fast migratng bands apparently dissociating to form slower-migrating bands. He, however, concluded that the phenomenon was caused by the dissociation of SDS from LPS during

electrophoresis. Peterson and McGroarty (1985) in their studies of the migration rates of the multimers concluded that the LPS multimers were either formed immediately before or during the electrophoresis. Logan and Trust (1984) have also reported aggregation of LPS which could be reversed by resolubilisation in SDS.

O-antigens of Ogawa NIH 41 and Inaba 35 A3 exhibited only a limited variation in migration rate. Consequently, an aggregate of the fastest moving band would be likely to appear well behind the slowest moving band of the main LPS pattern and aggregation is unlikely to have affected the work described here. Moreover, the process of aggregation is enhanced by high concentrations of LPS or low concentrations of SDS. The low concentrations of LPS used for the resolution studies in this work are unlikely to have favoured the formation of aggregates.

Consequently, the high molecular weight bands exhibited by the Inaba 569 B LPS may have been a genuinely unique structural feature or a result of aggregation as described by Peterson and McGroarty (1985) or a spontaneous aggregation (Section 6.7.2).

6.7.2 Aggregation of LPS on storage

Aggregation of LPS was observed in this study when solutions of it in distilled water were stored for long periods of time at 4^{0}C. Tsai and Frasch (1980) have also reported aggregation of LPS on long storage. Shands and Chun (1980) have shown that large aggregates of LPS are formed spontaneously and

can be partially dissociated by detergent. The aggregate size is dependent on both the detergent concentration and the type of detergent used. Hurlbert and Hurlbert (1977) have demonstrated the dissociation of LPS into subunits following treatment with detergents. The re-aggregation of the subunits was then achieved by removing the dispersing agent followed by freezing and thawing.

6.8 Immunoblotting of LPS

Bacterial LPS molecules from various organisms have been immunoblotted with polyclonal and monoclonal antibodies (Perez et al, 1985; Poxton et al, 1985; Sidberry et al, 1985; Ogasawara et al, 1985) and it has been observed that some LPS molecules have very characteristic profiles of migration in SDS-PAGE and Western blotting. A typical immunoblot of LPS would consist of a ladder shape as obtained by Sidberry et al (1985) with E. coli and Pseudomonas LPS. LPS from V. cholerae did not conform with the ladder shape exhibited by the O-antigens. Unresolved O-antigens of V. cholerae appeared as smears on immunoblotting (Fig. 4.3a and b). Since the MCAbs reacted with the whole region of the unresolved O-antigen this indicates that even if heterogeneity does exist in the antigen the MCAbs did not differentiate it. Immunoblots similar to that of V. cholerae LPS were obtained by Ogasawara et al, (1985) with Yersinia enterocolitica O:3 and by Bundle et al, (1984) with Y. enterocolitica O:9 and B. abortus O-antigens. The confluent pattern of the immunoblots was attributed to the chemical nature

of the O-antigenswhich were thought to be homopolysaccharides with no microheterogeneity. The O-side chains of B. abortus, Y. enterocolitica O:9 and V. cholerae have been proved to be polymers of monosaccharides (Section 1.18). Hence the feature of microheterogeneity in the LPS may be characteristic only of apparenthomopolymers, i.e. molecules with small differences in their O side chains.

The resolved LPS of V. cholerae also failed to exhibit periodic bands in an immunoblot because of problems with the detection systems. Basically, the enzyme detection system (Section 4.15.1) had the potential to differentiate between the bands but was not sensitive enough for the low concentrations necessary to resolve them. The Protein A system (Section 4.15.2) on the other hand, had the sensitivity to cope with the nanogram concentrations but gave blurred autoradiographs due to the long pathlength of the gamma rays emitted by the isotope. The experimental problem was further exacerbated by the lack of a sensitive stain for LPS which could be applied to nitrocellulose paper so that the fidelity of transfer of the closely knit bands from the gels could be assessed. After numerous experiments with the two detection systems, bands in the immunoblot, reminiscent of, but more diffuse than those in the original silver stain were demonstrable (Fig 4.10.b).

A common feature of the immunoblots of resolved LPS was the presence of differentially reacting regions. The fact that it appeared in both systems of detection ruled out the possibility of a methodological artefact. The extent of reaction of the

O-antigen did not decrease as it might be expected to have done with a decrease in the number of side chains, i.e. molecular weight (Fig. 4.9), and instead there was an apparently biphasic reaction with both the high and low molecular weight regions of the band reacting more than the median. Such a phenomenon could not be explained from the molecular structure of the O antigen. The alternative could be a selectively inefficient electrophoretic transfer of the resolved LPS which does not correlate with molecular weight or charge but depends on some other physicochemical factor operating during transfer.

There is only one report of immunoblotting of V. cholerae LPS in the literature and this is a recent paper from Sciortino et al (1985) who have produced 66 stable monoclonal antibodies from a single fusion, screened by immunoblotting. Those of their clones which reacted with LPS also did not show the typical ladder shape but resembled the immunoblot obtained in this study with Inaba 569 B. The diffused bands the authors presented clearly expressed heterogeneity in them. The widely spaced smears the authors obtained may have been due to LPS aggregates (Section 6.7).

Poxton et al, (1985) produced evidence of outer membrane protein linked LPS complexes in Pseudomonas aeroginosa. The MCAbs reactive with them recognised both the protein and LPS suggesting that the antibodies interacted with both. Protein antigens have been reported in V. cholerae outer membranes but they have not been associated with the LPS. The studies conducted here with V. cholerae LPS did not have any protein contaminants

as detected by coomassie blue or silver stain on the gel itself, or amido black on the blot. None of the six MCAbs reacted detectably with any protein in the vibrio outer membrane thus apparently ruling out the possibility of involvement of proteins in antibody-antigen interaction.However, in view of the fact that low affinity cross-reactions have been detected with heterologous LPS molecules (Section 6.10.7), a minor contribution of LPS-protein interactions cannot be ruled out in this study. Since it has not effectively been tested.

6.9 Other biological methods of analysis of V. cholerae LPS

While this study has been concentrated on immunological methods of detection of LPS antigens, other well established methods of analysis were tested and did not yield immediate useful information.

6.9.1 Lectin studies.

In this study, wheat germ agglutinin and concanavalin A did not show any binding with the V. cholerae LPS when assessed by either direct gel blotting or nitrocellulose blotting respectively (Section 4.12). However, Kabir (1982) reported precipitation of alkali treated LPS with con A and acetylated LPS with WGA (Section 4.13). The concentrations of LPS and lectins used for the assays were high and no negative controls were included in the data and so it seems very possible that the high concentrations of lectins and LPS could have led to non-specific precipitation. It is, however, also possible that the

interactions between the lectins and the LPS may have been weak and hence not suitable for detection by immunoblotting method employed in this study.

6.9.2 Enzyme studies

Sen et al, (1980) reported the presence of both alpha- and beta- anomeric configurations of the sugar residues in V. cholerae LPS. The enzymological studies undertaken here to investigate the nature of linkages of the sugar residues contained in the LPS (Section 4.11) were not successful due to the interference of the enzymes in the assessment of the migration of the LPS. In addition, the purity of the enzymes used for the study was suspect. All the enzymes employed were exoglycosidases and so might not have been expected to dissect the molecule internally but rather to trim the edges and sharpen the electrophoretic profile and this was clearly not possible.

6.10 Assay methods and the detection of MCAb cross-reactivity

Cross reactions among polyclonal antisera raised against bacterial antigens are common. They are, however, difficult to evaluate in terms of fine specificity because of the heterogeneity of the antibody population. In hybridoma technology, cross reactions may be expected to be simplified. Where the antibody reacts with identical epitopes on two populations of antigen, the cross reaction is likely to be more extensive than with conventional serum. Where the antibody reacts

with a unique epitope, cross reaction may be expected to be negligible. Conversely, however, when the whole antigen rather than a single epitope is considered, all the antibodies in polyclonal antisera have this target in common and a mature immune response should statistically favour the major immunising antigen. A homogeneous population of a single monoclonal antibody may be actually more able to make significant contact with antigens other than the immunising one and lead to equally complex cross reactions. The situation is further complicated in cases like this when the cross reacting antigens bear strong structural similarities.

The MCAbs raised in this study against V. cholerae organisms show varying degrees of cross-reactivity against different strains of bacteria. This anomalous behaviour of the MCAbs is dependent on the type of assay employed

6.10.1 ELISA

ELISA is the most widely used test system for the screening of emerging hybridomas. Firstly, it is a convenient assay for screening a large number of clones rapidly. Secondly, it is considered to be a sensitive test and yet involves the use of comparatively low amounts of antigen and antibody. ELISA has been modified by various workers to be used for inhibition studies of MCAbs with polyclonal or monoclonal reagents from a second species, for epitope detection of the antigen (Friguet et al, 1983) and for the quantitation of antibodies (Engvall et al, 1971). In this study ELISA has been mainly used for the

screening of hybridomas. Other studies such as competitive inhibition with LPS and the effect of antigen-antibody concentrations on specifities were also conducted on ELISA.

Initial screening of the MCAbs on ELISA as hybridoma supernatant on optimum antigen concentrations revealed that the established clones were either totally specific towards the Inaba or the Ogawa serotype. Conventional serology would classify the antibodies as anti-B or anti-C antibodies.

Competition ELISA was undertaken to verify the respective serotype specifities of the MCAbs by using LPS as the competing antigen (Section 5.1). However, even very high concentrations of heterologous LPS did not inhibit the MCAbs from binding to the vibrios of the homologous serotypes and the original classification of all six antibodies was confirmed by this procedure.

Once it had been established by other methods that some of the MCAbs were in fact cross-reactive under certain conditions, the six MCAbs were tested on ELISA with very large amounts of antigen coated on the plates. High concentrations of antigen changed the pattern of reactivity of three antibodies. I1A1, I4B1 and I4C3 clearly demonstrated their ability to bind to Ogawa vibrios, though the activity was always much lower than against Inaba vibrios. The detection of cross-reactivity on ELISA at only high concentrations of the Ogawa vibrios indicated that the binding between Ogawa vibrio and the Inaba series of antibodies was of a comparatively low affinity. O3D1 was the only antibody of the Ogawa series to cross react weakly and it required very

large amounts of not only the antigen, but also the antibody to do so, indicating that it had an even lower affinity for the heterologous serotype than the Inaba series of MCAbs.

The increase in antigen concentration which is necessary for the demonstration of cross reactivity can be simply interpreted (ignoring antibody bivalence temporarily) as pushing the equation

Ab + Ag --> AbAg

to the right. In molecular terms, when an antibody dissociates from an antigen and there are many other antigenic epitopes in the vicinity, it has a greater opportunity for reassociating with one of these. With histocompatability antigens, which provide an analogous, although not identical situation, the elegant work of Ways and Parham (1983) and Parham (1984) has divided such low affinity cross reacting antibodies into two classes which emphasise that bivalence cannot,in practice, be ignored:-

(1) Those antibodies in which the prime reason for low affinity is a rapid rate of dissociation

(2) Those antibodies in which the prime reason for low affinity is a low rate of association and where the rate of dissociation is also reasonably low.

In model 1 the antibody bivalence is of critical importance and avidity is much greater than affinity. In model two, there is very little difference between the two fragments. These studies were not conducted. However, the results of the competition ELISA would suggest that model 1 was a more likely interpretation for all four of the six cross reactive antibodies since in this

scheme it is possible to envisage antibodies weakly bound to the heterologous competing LPS able to dissociate rapidly and make a more stable contact with the plate bound homologous material, thus obliterating measurement of the valid but weak competition.

The main conclusions from the ELISA and competition ELISA results are therefore that, while all of the original six MCAbs may be made to appear totally serotype specific, four of them, namely the Inaba series and O3D1 (very weakly) may be shown to cross react with the heterologous V. cholerae serotype if the assay conditions are suitably manipulated. None can be shown to cross react with the other gram-negative bacteria tested including B. abortus, at the the highest practicable antigen concentrations.

6.10.2 Immunoblotting

Immunoblotting may be considered similar to ELISA as an assay procedure, especially where an enzyme blot is employed and may be expected to give a similar pattern of cross reactions. However, the antigen has been dissected on SDS-PAGE and such a procedure may alter both its structure and its relative concentration to an unknown extent. The concentrations employed in this study were necessarily low in order to achieve fine resolution of the bands (Section 4.14.2) and the specificity profiles of all six antibodies fitted their original Inaba specific or Ogawa specific classification with no cross reactions being detectable. The MCAbs showed no tendency to blot with protein components of the bacteria. The main value of the

experiment was therefore to confirm that the major epitopes of all six antibodies were present on the LPS molecule alone. Immunoblotting has also been used by Sciortino et al (1985) and Ogasawara et al (1985) for the determination of the specificity of MCAbs raised against bacterial LPS.

6.10.3 Radiolabelled immunoglobulin binding assays

MCAbs were labelled with $Na^{125}I$ in order to measure primary antibody-antigen interactions without the involvement of a second antibody. This removes potential complications which could have been introduced by variation in quality or labelling of the second antibody in ELISA or immunofluorescence and also zone effects which can be observed with bacteria when the binding of the first antibody is so close as to impede the interaction of the second. Direct binding assays with labelled antibodies also detected high affinity attachments only and did not reveal the extent of cross-reaction taking place as observed in, say, agglutination tests. The anti-Inaba antibodies bound very weakly to the Ogawa vibrios. However, the inhibition assays with cold anti-Ogawa antibodies confirmed that the binding was to a similar or neighbouring epitope. In the inhibition studies complete displacement of any labelled antibody by cold antibody was not achieved and this may be because saturating amounts of cold antibody were not used. This assay confirmed the ELISA data with respect to cross reactivity of the Inaba series of MCAbs with Ogawa vibrios.

6.10.4 Indirect Immunofluorescence Assay

This assay could again be expected to yield a similar pattern of cross reactivity to the ELISA assay. It is however possible to measure the extent to which binding is uniform within and among organisms in the population under test or whether, for example, binding is cooperative. Cross-reaction of the anti-Inaba antibodies with the Ogawa vibrios was, clearly demonstrated in the indirect immunofluorescence assay although washing was included in the protocol as for ELISA and direct binding assays. Several factors may have influenced the results obtained in this test system. Firstly, the commercial preparation and label of the second antibody was different from that used in ELISA or immunoblotting. Secondly, the antigens were fixed with alcohol thus minimising leaching, and possibly altering epitope availability. Thirdly, greater amounts of antigen were presented to the antibodies (Section 2.3.3.3) than in ELISA assays (Section 2.3.3.1),so that the assay was more equivalent to the ELISA involving high antigen concentrations. Finally, PBS as wash buffer may not have exerted as strong a dissociating force as PBS-Tween in the weak interactions between antigen and antibody. The very weak activity of O3D1 against Inaba was not detected in this test. The higher reactivity of the Ogawa positive MCAbs against homologous organisms compared to that of Inaba positive MCAbs against homologous organisms was consistently observed and may relate to the ease of accessibility of the primary or secondary antibody.

6.10.5 Agglutination tests

Agglutination is the most frequently employed test for the identification of strains of micro-organisms with standard polyclonal antibodies or, conversely for the determination of the specificity of polyclonal antibodies using known strains of bacteria. In order to determine the specifities of MCAbs by agglutination, ascitic fluid or ammonium sulphate concentrated antibodies are generally used. The quantities of antigen required for agglutination are also higher than for any other immunological assay, and hence low affinity interactions may play a role in agglutination tests. In spite of the drawbacks it is still the most widely used assay because of its simplicity.

Agglutination tests are less easy to analyse than ELISA type assays. The antibody must not only bind to the bacterium, but also bind in a manner which makes it geometrically possible to form a bridge between two neighbouring bacteria with enough free energy to counteract diffusion forces. Factors such as epitope position and density and cooperativity of binding thus determine the extent to which a single MCAb can agglutinate a population of bacteria.

The activity of the anti-Inaba antibodies against B. abortus organisms and O3D1 against Inaba were observed only in this test and the complement fixation. O3D1 did not agglutinate Ogawa vibrios on its own but could agglutinate Inaba vibrios. O4A6 and O4D2 were not capable of agglutinating either the homologous or the heterologous serotypes of bacteria despite

their high ELISA titres. Bundle et al, (1984) also reported negative agglutination properties of a MCAb of isotype IgM although it exhibited a high ELISA titre.

Agglutination tests proved to be valuable in this study for revealing weak antigen-antibody interactions not detected in other assays. Results from this study suggest that agglutination tests can detect both high and low affinity interactions. This is presumably because the antigen remains suspended in a solution of antibody without washing procedures to remove unbound material, and also because of the very high concentration of each of the two components.

The nonagglutinating properties of O4D2 and O4A6 despite their high reactivity towards the Ogawa vibrios may reflect the position of the binding site rather than the affinity of the antibody which binds strongly in all other tests.

6.10.6 Complement fixation assay

The complement fixation assay is another one in which washing is not involved and where low affinity interactions may therefore be detected. However, it is carried out at much lower concentrations of antibody and antigen than the agglutination assay. The detection system does not require that the antibody cross links two bacteria but instead, requires that the antibody be of a class which fixes complement. In addition, it is often suggested that two IgG molecules must bind in close proximity in order to fix complement (Roitt et al, 1985) and so the cross reaction pattern might well be expected to be different. In

practice, the two non-washing assays were similar except that all the Ogawa series of antibodies, which failed to agglutinate the homologous vibrios, fixed complement with homologous antigen as well as displaying the cross reactions observed on agglutination. The interaction of the anti-Inaba antibodies with Ogawa vibrios and B. abortus organisms were clearly demonstrated, and it was interesting to observe the activity of I4C3 gradually decrease as it was simultaneously tested against different strains of bacteria. The preference for Inaba was about three times than that for Ogawa and around 60 times that for B. abortus (Table 5.9a). 03D1 showed reaction with Inaba only at 80 fold the concentration where it reacted with homologous antigen (Table 5.9b) and yet it preferentially agglutinated the Inaba vibrios in primary agglutination. Observations of this type were reported by Bundle et al, (1984). One of their anti-Y. enterocolitica MCAbs of isotype IgG2a agglutinated B. abortus but not Y. enterocolitica.

6.10.7 Classification of the six anti-V.cholerae antibodies

The variety of immunological assays conducted in this study revealed fine differences in the panel of antibodies raised against the two serotypes of V. cholerae. The behaviour of the antibodies throughout the study led to their classification into three separate groups, determined by the ratio of reactivity between the two serotypes in any particular assay, since in most cases tissue culture supernatant rather than pure antibody was used for the test and the immunoglobulin content could vary from

batch to batch.

Group one antibodies comprised I1A1, I4B1 and I4C3, generated from animals immunised with the Inaba serotype, shown by initial screening to be specific to the Inaba serotype but subsequently shown to cross react with the Ogawa serotype at a ratio of 1/2-1/4 of homologous activities on other assays. All of this group showed cross reactivity with Brucella abortus on agglutination and the only one tested on complement fixation (I4C3) showed cross reaction with B. abortus. All three are therefore taken to be similarly cross reactive for the purposes of discussion, although further analysis with B. abortus and other gram-negative bacteria may subdivide this group.

Group two consisted of O4D2 and O4A6, generated from animals immunised with the Ogawa serotype and shown by initial screening to be specific to the Ogawa serotype. In secondary antibody tests, it was very strongly positive while in direct labelled antibody tests, it showed less binding than the group 1 antibodies, this could relate to the loss of antibodies or antibody activity during the multiple purification steps. These antibodies were non-agglutinating on all organisms tested.

Group three is the single antibody, O3D1 which was generated from animals imunised with the Ogawa serotype and originally classified as Ogawa specific. It could be shown, by the two non-washing assays to cross react with Inaba vibrios, but not any other organism tested. One feature of interest of this antibody, is that it agglutinates Inaba but not Ogawa vibrios although its reaction with Inaba vibrios is much weaker.

To this list, must be added the MCAbs of Gustafsson and Holme (1983) which were studied by exchange. In the two tests performed on these, ELISA and agglutination, the three antibodies generated by Gustafsson and Holme, behaved exactly as expected for antibodies reacting with the classical A, B and C antigens. It was of particular interest to note that all three agglutinated the appropriate serotypes at a concentration of ascites fluid which gave a much lower ELISA titre than was determined with the antibodies used in this study. However, even their A specific antibody was not equally reactive with both serotypes as undertaken in this study. Thus, the classical antigens of V. cholerae can, with minor caveats, also be demonstrated by hybridoma technology.

6.11 Dissection of V.cholerae antigens with MCAbs of three different idiotypes

Detailed studies of the relationship between an antibody making a high affinity contact with a complex antigen have not been available until the advent of hybridoma technology. The earlier X-ray crystallographic data on interactions and cross reactions (Richards et al,1975) show that contact points may be made between an antibody of a myeloma cell line and two or more shelf selected antigens, each of totally different structure. When hybridoma technology was developed, the early detailed studies of selected interactions were with small haptens and it soon became apparent that the antibodies raised could see much

deeper into the immunising mixture beyond the hapten structure and into the carrier material (Rodwell et al, 1983). A detailed crystallographic study of the reaction of an antibody with an antigen is that of Amit et al, (1985) who analysed the interaction of a MCAb generated and selected against hen egg white lysosyme with its antigen. There are many contact points beyond the areas previously envisaged.

Chemical analyses have shown that Inaba and Ogawa LPS molecules are very similar with the possible exception of one sugar residue. The Ogawa serotype has been reported to contain the sugar 4-amino-4-deoxy-arabinose and the Inaba LPS to lack it (Redmond, 1978). Kenne et al, (1982) suggested that the polymer of amino acylated perosamine residues common to both serotypes probably represents the 01 antigen of V. cholerae LPS. The precise nature of Ogawa or Inaba specific antigens is still obscure and the contribution of 4-amino-4-deoxy-arabinose towards the type specific antigen of Ogawa vibrios remains speculative. However, the data obtained from SDS-PAGE suggests that this sugar may not be a regular feature of the repeating O antigen in the Ogawa strain alone since the ladder pattern does not accommodate such a hypothesis. (Section 6.6.1).

The idiotypes of the MCAbs established in this study do not conform to the A, B and C antigens defined for V. cholerae organisms by classical serology. Group one antibodies, specific against the Inaba serotype in certain assays (Section 5.1) bind to Ogawa vibrios as well but with a lower affinity and thus cannot be classified as anti-A or anti-C antibodies. It is clear

that these antibodies see a similar epitope on the Ogawa vibrio. A similar but not identical epitope may well attract an antibody as long as some contacts can be made (Amit et al, 1985).

The Ogawa and Inaba LPS molecules share not only similar O-chains but also a common core (Hisatsune and Kondo, 1980; Brade, 1985). The outer membrane proteins of the two serotypes have also been shown to be similar (Kabir, 1980; Manning and Haynes, 1984). Hence, the common epitopes recognised by I1A1, I4B1 or I4C3 may involve interactions with the core and/or membrane protein antigens. The region linking of the O-side chain to the core has been identified as an epitope recognised by two anti-Y. enterocolitica MCAbs established by Bundle et al, (1984).

The data obtained from the six MCAbs in this panel cannot be easily interpreted in terms of subtle fine structural differences in the LPS of the two strains for a variety of reasons. Firstly, the structure of the LPS is not known (Section 1.13.2), secondly, the cross reactive panel represents only three basic types of specificity and more antibodies with yet further specificity profiles would probably be required to obtain more data from the antibodies alone. Thirdly, the size of the repeat region in V. cholerae is small as shown by the microheterogeniety studies on gels. Thus, when interpreting interactions it is necessary to consider the possibility that the idiotype of any antibody may encompass more than one repeating unit in non integral fashion. Finally, all the cross reactions demonstrated were shown on whole vibrios and as such may involve

proteins as well as core components of the LPS. The antigen and the potential contribution of proteins cannot be ruled out until tests utilising LPS alone have also demonstrated cross reactivity. The involvement of the core LPS in the cross-reactions seem likely because the group one antibodies can agglutinate B. abortus but not Y. enterocolitica organisms and these two organisms differ only in the core structure of their LPS.

Some attempt may be made to discuss the types of interactions which may be involved between antibody and bacterial LPS antigen. Schalch et al, (1979) and Cisar et al, (1975) have demonstrated that the ability of antibodies to precipitate polysaccharides is associated with the multivalency of the antigens. A linear streptococcal polysaccharide was shown to bind to one type of antibody (which was an agglutinating antibody) in the ratio of 1:4 due to the presence of several binding sites on the linear portion of the polymeric molecule. A second type of antibody bound in the ratio of 1:1 and did not agglutinate and was assigned to interaction with the terminal reducing end of the polysaccharide (Schalch et al, 1979).

A similar situation could be envisaged in this case. I1A1, I4B1 and I4C3 could correspond to the agglutinating type of antibody. The vibrio LPS molecule is effectively a homopolysaccharide and by analogy may offer binding sites along the linear extension of the molecule. As discussed above, (Section 6.6.1) the chain length of V. cholerae LPS is not known but from SDS-PAGE studies it is clear that the mean chain

length is much above one repeat unit and the molecule thus possesses several potential antibody binding sites. The multivalent binding could thus precipitate the organisms. Conversely, the group two antibodies, O4D2 and O4A6, could exhibit chain end specifities because they are nonprecipitating antibodies. Cisar et al (1975) have shown that the interaction of mouse myeloma proteins with dextrans emphasises the immunodominancy of terminal nonreducing -linked glycosyl residue of linear homopolysaccharides. Since O4D2 and O4A6 do not bind to Inaba as was established by several assays, this would imply differences in the terminal groups of the two serotypes.

This interpretation of the group one antibodies binding to internal regions along the length of the LPS while group two bind to the chain ends, is simple and attractive. However, it becomes hard to sustain this hypothesis when the group three antibody, O3D1 is considered. One would then have to suggest that this antibody binds to terminal residues on Ogawa but internal ones on Inaba. Moreover, the displacement of group one antibody's cross-reaction by group two and three antibodies (Section 5.14.3) cannot be easily explained by this theory. Thus the interpretation of Schalch et al, (1979) cannot be readily applied to this situation.

6.12 The significance of cross reactive antibodies in applied and commercial situations

6.12.1 In vivo applications (i) Applications for cholera therapy

The use of the antibodies produced in this study *in vivo* would present several complications. Not only are they foreign to man and liable to rejection if employed over a long period of time, but they are also basically required in the intestinal tract and the access of such antibodies after intravenous injection may well be highly limited since IgA antibodies with their own complex transport pathway through the intestinal epithelium, may be the only major class of antibody to be effective in this particular disease.

6.12.2 In vivo applications (ii) Implications of cross reactions for general therapy

Monoclonal antibodies are considered as possible agents for therapy of both tumours and autoimmune conditions. Where *in vivo* use is contemplated, the antibody cannot be screened by a method equivalent to its final application and consequently screening is generally effected by ELISA or immunocytochemical assays on isolated tumour cells. Such assays will only detect the higher affinity contacts described in this work, and the actual environment in which the antibody is expected to operate is poorly understood. Thus, any antibody employed in therapy against an apparently specific tumour antigen, may have considerable

cross reactive potential.

6.12.3 Implications for in vitro applications. Diagnostic tests

In monoclonal antibody technology, the importance of selecting the emerging hybridoma by the assay in which the monoclonal antibody will be finally employed is well known. For diagnostic tests, the type of assay which has been popular for many antigens has been an ELISA -like one which involves the use of two monoclonal antibodies. One is fixed on a solid phase support such as a stick or filter and is reacted with the fluid which may contain the antigen. The second is labelled with an enzyme and used to detect the binding of antigen to the first monoclonal. With protein molecules, the two monoclonals need to have different epitopes whereas with bacteria, this is a less stringent requirement because of antigen multivalence. In such assays, the monoclonal antibodies generated in this study should perform with true serotype specific fidelity as suitable diagnostic reagents. However, tests of this type are still comparatively costly and there is also considerable pressure for the production of monoclonal antibodies which may be used in simple agglutination tests in the field in the third world with clinical samples with variable concentrations of antigen. Monoclonal antibodies to the major blood group antigens are screened in this way because they will be used in this way for testing (Fraser et al, 1984) and such antibodies have proved to be highly successful, possibly in part because of the high antigen density in an easily obtained sample of red blood cells.

None of the antibodies produced in this study would have shown cross reaction on screening but if used as concentrate for field testing, would have produced exceedingly confusing results. Much commercial pressure is directed towards sensitivity which is readily achieved in ELISA and much also towards convenience and cost which favour agglutination type assays.

Cross reactions are usually regared as a negative feature of monoclonal antibodies. However, as Mossman et al, (1980) point out, they may also be adapted so that a single monoclonal antibody may be used for many applications. Thus the group one series of antibodies in this study could be used as either V. cholerae 01 or Inaba specific reagent according to assay conditions. Similarly, 03D1 could be used as Ogawa specific on ELISA and Inaba specific on agglutination tests. Thus, assay dependent cross reactivity, properly understood can not only be employed to analyse antigenic determinants on complex bacterial surfaces but also to provide versatile diagnostic reagents.

REFERENCES

Afzal, M., Tengerdy, R.P., Squire, P.G. and Ellis, R.P. (1984) J. Clin. Microbiol. 20, 1159-1164.

Ahvonen, P. and Sievers, K. (1969) Acta Med. Scand. 185, 121-125.

Alt, F., Rosenberg, N., Casanova, R., Thomas, E. and Baltimore, D. (1982) Nature 296, 325-330.

Amit, A.G., Mariuzza, R.A., Phillips, S.E.V. and Poljak, R.J. (1985) Nature, 313, 156-158.

Andersson, J., Sjoberg, O. and Moller,G. (1972) Eur. J. Immunol. 2, 349.

Baenziger, J.U. and Fiete, D. (1979) J. Biol.Chem. 254, 2400-2407.

Barua, D., and Paguio, A.S. (1977) Ann. Human. Biol. 4, 489-492.

Barua, D. and Watanabe,Y. (1972) J. Hyg. Camb. 70, 161-169.

Batteiger, B., Newhall, W.J.V. and Jones, R.B. (1982) J. Immunol. Meth. 55, 297-307.

Bjorklund, M. and Coutinho, A. (1983) Eur. J. Immunol. 13, 44-50.

Bloom, A.D. and Nakamura, F.T. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 2689-2692.

Brade, H. (1985) J. Bacteriol. 161, 795-798.

Brade, H. and Galanos, C. (1983) Anal. Biochem. 132, 158-159.

Brade, H. and Galanos, C. (1983) Infect. Immun. 42, 250-256.

Brade, H. and Rietschel, E.T. (1984) Eur. J. Biochem. 145, 231-236.

Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.

Bruck, C., Portetelle, D., Gilneur, C. and Bollen, A. (1982) J. Immunol. Meth. 53, 313.

Bruins, S.C., Stumacher, R., Johns, M.A. and McCabe, W.R. (1977) Infect.Immun. 17, 16-20.

Bundle, D.R., Gidney, M.A.J., Perry, M.B., Duncan, R. and Cherwownogrodzky, J.W. (1984) Infect. Immun. 46, 389-393.

Burnet, F.M. (1959) In The Clonal Selection Theory of Acquired Immunity. Cambridge University Press.

Cadieux, J.E. Kuzid, J., Milazzo, F.H. and Kopinski, A.M. (1983) J. Bacteriol. 155, 817.

Calame, K.L. (1985) Ann. Rev. Immunol. 3, 159-195.

Campbell, A.M. (1984) In Laboratory Techniques in Biochemistry and Molecular Biology vol.13 Ed. Burdon, R.H. and Knippenberg, P.H.van, Elsevier.

Caroff, M., Bundle, D.R. and Perry, M.B. (1984) Eur. J. Biochem. 139, 195-200

Caroff, M., Bundle, D.R., Perry, M.B., Cherwonogrodzky, J.W. and Duncan, J.R. (1984) Infect. Immun. 46, 384-388.

Cash, R.A., Music, S.I., Libonati, J.P., Craig, J.P., Pierce, N.F. and Hornick, R.B. (1974) J. Infect. Dis. 130, 325-333.

Cassel, D. and Selinger, Z. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 3307-3311.

Chaudhuri, A. (1977) Lancet 2, 404-405.

Chaudhuri, R.N. (1971) Trop. Doctor 1, 5.

Cisar, J., Kabat, E.A., Dorner, M.M. and Lino, J. (1975) J. Exp. Med. 142, 435-459.

Clark, M., Cobbold, S., Hale, G. and Waldmann, H. (1983) Immunol.Today 4, 100-102.

Clements, M.L., Levine, M. M., Young, C.R., Black, R.E., Lim, Y.L., Robins-Browne, R.M. and Craig, J.P. (1982) J. Infect. Dis. 145, 465-473.

Corbel, M.J., Cockrem, D.S. and Brewer, R.A. Third International Symposium on Brucellosis, Algiers, Algeria, (1983)

Cryz, S.J.Jr., Furer, E. and Germanier, R. (1982) J, Clin. Microbiol. 16, 41-45.

Cuatrecasas, P. (1973) Biochem. J. 12, 3547-3558.

De, S.N. (1959) Nature 183, 1533-1534

Dlabac, V. (1969) Collq. Int. Clin. Notes. Respir. Dis. 174, 297-305.

Dubray, G. and Bezard, G. (1982) Anal. Biochem. 119, 325-329.

Dutt, A.K., Alwi, S. and Veluthan, T. (1971) Trans. R. Soc. Trop. Med. Hyg. 65, 815-818.

Dutta, N.K., Panse, M.V. and Kulkarni, D.R. (1959) J. Bacteriol. 78, 594-595

Eisele, C.W., McCullough, N.B., Beal, G.A. and Burrows, W. (1946) Proc. Soc. Exp. Biol. Med. 61, 89-91.

Engvall, E., Jonsson, K. and Perham, P. (1971) Biochim. Biophys. Acta 251, 427-434.

Feeley, J.C. (1969) J. Bacteriol. 99, 645-649.

Finkelstein, R.A. (1973) CRC Critical Rev. Microbiol. 2, 553-623.

Finkelstein. R.A. and LoSpalluto, J.J. (1969) J. Exp. Med. 130, 185-202.

Frankel, M.E. and Gerhard, W. (1979) Mol. Immunol. 16, 101.

Fraser, R.H., Allan, E.K., Inglis, G., Munro, A.C., Mackie, A. and Mitchell, R. (1984) Exp. Clin. Immunogenet. 1, 145-151.

Freter, R. and Gangarosa, E.J. (1963) J. Immunol. 91, 724-729.

Friguet, B., Djavadi-Ohaniance, L., Pages, J., Bussard, A. and Goldberg, M. (1983) J. Immunol. Meth. 60, 351.

Fujita, K. and Finkelstein, R.A. (1972) J. Infect. Dis. 125, 647-655.

Galanos, C., Luderitz, O. and Westphal, O. (1971) Eur. J. Biochem. 24, 116-122.

Galanos, C., Luderitz, O., Rietschel, E.T. and Westphal, O. (1977) in International Review of Biochemistry, Biochemistry of lipids II, Ed. Goodwin, T.W. University Park Press, vol. 14, 239-335.

Gallut, J. (1953) Ann. Inst. Pasteur. (Paris) 85, 261-262.

Gangarosa, E.J., and Mosley, W.H. (1975) In Cholera, Ed. Barua, D. and Burrows, W. pp 381-403.

Ganguly, R., Clem, L.W., Benck, Z., Sinha, R., Sakazaki, R. and Waldham, R.H. (1975) Bull. W. H. O. 52, 323-330.

Gardner, A.D. and Venkatraman, K.V. (1935) J. Hyg. 35, 262-282.

Glass, R.I., Becker, S., Huq, M.I., Stoll, B.J., Khan, M.U., Merson, M.H., Vee, J.V. and Black, R.E. (1982) Am. J. Epidemiol. 116, 959-970.

Glass, R. I., Holmgren, J., Haley, C. E., Khan, M. R.,

Svennerholm, A.M., Stoll, B.J., Hossain, K.M.B., Black, R.E., Yunus, M. and Barua, D. (1985) Am. J. Epidemiol. 121, 791-796

Goding, J.W. (1978) J. Immunol. Meth. 20, 241-253.

Grossman, N. and Leive. L. (1984) J. Immunol. 132, 376-385.

Goldamn, C. and Leive. L. (1980) Eur. J. Biochem. 107, 145-153.

Gustafsson, B. and Holme, T. (1983) J. Clin. Microbiol. 18, 480-485.

Gustafsson, B., Rosen, A. and Holme, T. (1982) Infect.Immun. 38, 449-454.

Hames, B.D. and Rickwood, D. (1981) In gel electrophoresis of proteins. IRL Press London.

Hammond, S.M., Lambert, P.A. and Ryecroft, A.N. (1984) In The Bacterial Cell Surface, Croom Helm, London and Sydney.pp 60-72.

Hisatsune, K. and Kondo, S. (1980) Biochem. J. 185, 77-81.

Hisatsune, K. and Kondo, S. (1980) Biochem. J. 185, 77-81.

Hitchcock, P.J. (1983) Eur. J. Biochem. 133, 685-688.

Hitchcock, P.J. and Brown, T.M. (1983) J. Bacteriol. 154, 269-277.

Holmgren, J. (1981) Nature 292, 413-417.

Holmgren, J. and Svennerholm, A.M. (1977) J. Infect. Dis. 136 (Suppl.), S105-S112.

Holmgren,J J., Lonnroth, I. and Svennerholm, L. (1973) Infect. Immun. 8, 208-214.

Honda, T. and Finkelstein, R.A. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 2052-2056.

Hurlbert, R.E. and Hurlbert, I.M. (1977) Infect. Immun. 16, 983-994.

Jackson, G.D.F and Redmond, J.W. (1971) FEBS Lett. 13, 117-120.

Jann, B., Jann, K. and Beyaert, G.O. (1973) Eur. J. Biochem. 37, 531-534.

Jann, B., Reske, K. and Jann, K. (1975) Eur. J. Biochem. 60, 239-246.

Johns, M.A., Bruins, S.C. and McCabe,W.R. (1977) Infect. Immun. 17, 9-15.

Jones, G.W. and Freter, R. (1976) Infect. Immun. 14, 240-245.

Jones, S., Chen, Y.-W., Isakson, P., Layton, J., Pure, E., Word, C., Kramer, P., Tucker,P. and Vitetta, E. (1983) J. Immunol. 131, 3049-3051.

Kabir, S. (1982) Infec. Immun. 38, 1263-1272.

Kabir, S. (1980) J. Bacteriol. 144, 382-389.

Kaper, J.B. and Levine, M.M. (1981) Lancet 2, 1162-1163.

Kaper, J.B., Baldini, M.M., Lockman, H.A. and Levine, M.M. (1983) Abstr. Annu. Meet. Am.Soc. Microbiol., B55, pp32.

Kato, N., Ohta,M., Kido,N., Naito,S., Nakashima, I., Nagase, F. and Yokochi, T. (1985) Med. Microbiol. Immunol. 174, 1-14.

Kauffmann, F. (1950) Acta. Pathol. Microbiol. Scand. 27, 283-299.

Kaur, J. and Shrivastava, J.B. (1965) in Proceedings of the Cholera Research Symposium, Honolulu, Hawaii, U.S. Public Health Service Publ. No. 1328.

Kelly, J.T. and Parker, C.D. (1981) J. Bacteriol. 145, 1018-1024.

Kenne, L., Lindberg, B., Unger, P., Gustafsson, B. and Holme, T. (1982) Carb. Res. 100, 341-349.

Kido, N., Ohta,M., Ito, H., Naito, S., Nagase, F., Nakashima, I. and Kato, N. (1985) Cell. Immunol. 91, 52-59.

King, C.A. and Heyningen, W.E.van (1973) J. Infect. Dis. 127, 639-647.

Kohler, G. and Milstein, C. (1975) Nature 256, 495-497.

Kohler, G. and Milstein, C. (1976) Eur. J. Immunol. 6, 511-519.

Kronvall, G. (1973) J. Med. Microbiol. 6, 187-190.

Lad, P.M., Nielson, T.B., Preston, N.S. and Rodbell, M. (1980) J. Biol. Chem. 255, 988-995.

Laemmli, U.K. (1970) Nature 227, 680-685.

Lam, G.T., Mandle, R.J. and Goodner, K. (1955) J. Infect. Dis. 97, 268-273

Levine, M.M., Black, R.E., Clements, M.L., Cisneros, L., Nalin, D.R. and Young, C.R. (1981) J. Infect. Dis. 143,

818-820.

Levine, M.M., Black, R.E., Clements, M.L., Lanata, C., Sears, S., Honda, T., Young, C.R. and Finkelstein, R.A. (1984) Infect. Immun. 43, 515-522.

Levine, M.M., Black, R.E., Clements, M.L., Nalin, D.R., Cisneros, L. and Finkelstein, R.A. (1981) In volunteer studies in development of vaccines against cholera and enterotoxigenic E. coli: a review, 443-459. Ed. Holme, T., Holmgren, J., Merson, M. and Mollby, R. Elsevier, Amsterdam.

Levine, M.M., Kaper, J.B., Black,R.E. and Clements,M.L. (1983) Microbiol. Rev. 47, 510-521.

Levine, M.M., Nalin, D.R. and Rennels, M.B. (1979) Ann. Human. Biol. 6, 369-374.

Levine, M.M., Nalin, D.R., Craig, J.P., Hoover, D., Bergquist, E.J., Pierce, N.F. and Libonati, J.P. (1979) Trans. R. Soc. Trop. Med. Hyg. 73, 3-9

Levine, M.M., Woodward, W.E., Formal, S.B., Gemski, P.,Jr., DuPont, H.L., Hornick, R.B. and Snyder, M.J. (1977) J. Infect. Dis. 136, 577-582.

Levine, R. and Nalin, D. (1976) Lancet 2, 1305

Lind, S.M., Carlin,N.I.A. and Lindberg, A.A. (1985) FEMS Microbiol. Lett. 28, 45-49.

Littlefield, J.W. (1964) Science 145, 709-710.

Logan, S.M. and Trust, T.J. (1981) Infect. Immun. 45, 210-216.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

Luderitz, O., Galanos, C., Lehman, V., Nurimen, M.M., Rietschel, E., Rosenfelder, G., SImon, M. and Westphal, O. (1973) J. Infect. Dis. 128, S17.

Luderitz,O., Galanos, C., Risse, H.J., Rucshmann, E., Schlecht, S., Schimdt, G., Schulte-Holthausen, H., Wheat, R., Westphal, O. and Schlosshardt, J. (1966) Ann. N.Y. Acad. Sci. 133, 349-374.

Mains, P., Sibbley,C. (1982) J. Immunol. 128, 1664-1670.

Majumdar, A.S. and Ghose, A.C. (1982) Infect.Immun. 36, 962-965.

Majumdar, A.S. and Ghose, A.C. (1981) Infect. Immun. 32, 9-14.

Majumdar, A.S., Dutta, P. and Ghose, A.C. (1981) Infect. Immun. 44, 439-444.

Majumdar, M., Mukherjee, A.K., Guhathakurta, B., Dutta, A. and Sasmal, D. (1982) Carb. Res. 108, 269-278.

Manning, P.A. and Haynes, D.R. (1984) FEMS Microbiol. Lett. 24, 297-302.

Manning, P.A., Imbesi, F. and Haynes, D.R. (1982) FEMS Microbiol. Lett. 14, 159-166.

Mansheim, B.J., Onderdonk, A.B. and Kasper, D.L. (1978) Immun. 120, 72.

Marcu, K.B. (1982) Cell 29, 719-721.

Marks, M.I., Ziegler, E.J., Douglas, H. (1982) J. Clin. Invest. 69, 742-749.

Mason, D.W. and Williams, A.F. (1980) Biochem. J. 1-20.

Mathur, T.N. (1960) Indian J. Med. Res. 48, 309-314.

McCabe,W.R., Scott, B.C., Craven, D.E. and Johns, M. (1977) J. Infect. Dis. 136, S161-S166.

McConell, M. and Wright, A. (1979) J. Bacteriol. 137, 746-751.

McCullough, N.B., Eisele, C.W. and Beal, G.A. (1948) J. Infect. Dis. 83, 55-59.

McDonald, I.J. and Adams, G.A. (1971) J. Gen. Microbiol. 65, 201-207.

McNamara, M.K., Ward, .R.E. and Kohler, H. (1984) Science 226, 1325.

Mekalanos, J.J., Swartz, D.J., Pearson, G.D.N., Harford, N., Groyne, F. and deWilde, M. (1983) Nature 306, 551-557.

Melchers, F. and Andersson, J. (1984) Cell 37, 715-720.

Milstein, C., Wright, B. and Cuello, A.C. (1983) Mol. Immunol. 20, 113.

Morrison, D.C. and Ryan, J.L. (1970) Adv. Immunol. 28, 293-450.

Mosley, W.H., Aziz, A.K.M., Rahman, A.S.M.M., Chowdhury, A.K.M.A. and Ahmed, A. (1973) Bull. W. H. O. 49, 381-387.

Mosley, W.H., Benson, A.S. and Barui, R. (1968) Bull. W.H.O. 38, 327-334.

Mosmann, T.R., Gallatin, M. and Longenecker, B.M. (1980) J. Immunol. 125, 1152-1156.

Moss, J., Manganiello, V.C. and Vaughan, M. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 4424-4427.

Mourant, A.E. and Kopec, A.C. (1978) In Blood groups and diseases, Oxford University Press.

Muschel, L.H. and Larsen, L.J. (1970) Proc. Soc. Exp. Biol. Med. 133, 345-348

Mutharia, L.M., Crockford, G., Bogard, W.C.Jr. and Hannock, R.E.W. (1984) 45, Infect.Immun. 631-636.

Nelson, K.J., Mather, E.L. and Perry,R.P. (1984) Nucleic Acids Res. 12, 1911-1922.

Nelles, M.J. and Niswander, C.A. (1984) Infect. Immun. 46, 677-681.

Nelson, B.W. and Roantree, R.J. (1967) J. Gen. Microbiol. 48, 179-188.

Neoh, S.H. and Rowley, D. (1971) Aust. J. Exp. Med. Sci. 49, 605-612.

Neoh, S.H. and Rowley, D. (1970) J.&Infect. Dis. 121, 505-513.

Nilsson, R., Myhre, E., Kronvall, G. and Sjogren, H.O. (1982) Mol. Immunol. 19, 119-126.

Ogasawara, M., Granfors, K., Kono, D.H. Hill. J.L. and Yu, D.T.Y. (1985) J. Immunol. 135, 553-559.

Ouchterlony, O. and Nilsson, L.A. (1978) In Handbook of Experimental Immunology, Ed. Weir,D.M. Chapter 19. Blackwell Scientific Publications Oxford.

Palva, E.T. and Makela, P.H. (1980) Eur. J. Biochem. 107, 137-144.

Parham, P. (1984) J. Biol. Chem. 259, 13077-13083.

Peavy, D.L., Shands, J.W., Alder, W.H. and Smith, R.T. (1973) J. Infect. Dis. 128, 591.

Perez, G.I.P., Hopkins, J.A. and Blaser, M.J. (1985) Infect.Immun. 48, 528-533.

Peterson, A.A. and McGroarty, E.J. (1985) J. Bacteriol. 162, 738-745.

Peterson, G.L. (1979) Anal. Biochem. 100, 201-220.

Peterson, J.W., Hejtmancik, K.E., Markel, D.E., Craig, J.P. and Kurosky, A. (1979) Infect. Immun. 24, 774-779.

Philippenes Cholera Committee (1973) Bull. W. H. O. 49, 13-19.

Pierce, N.F. (1978) J. Exp. Med. 148, 195-206.

Pierce, N.F. and Gowans, J.L. (1975) J. Exp. Med. 142, 1550-1563.

Pierce, N.F., Craig, W.C.Jr. and Sacci, J.B.Jr. (1982) Infect. immun. 37, 687-694.

Pierce, N.F., Sack, R.B. and Mahalanabis, D. (1970) In Principles and practice of cholera control. Publ. Hlth. Papers. W. H. O. No. 40. pp61.

Pollitzer, R. (1959) In Cholera, W. H. O. Geneva.

Poxton, I.R., Bell, G.T. and Barclay, G.R. (1985) FEMS Microbiol. Lett. 27, 247-251.

Quinn, R., Campbell, A.M. and Phillips, A.P. (1984) J. Gen. Microbiol. 130, 2285-2289.

Raetz, c.R.H., Purcell, S. and Takayama, K. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 4624-4628.

Raziuddin, S. (1978) Immunochem. 15, 611-614.

Raziuddin, S. (1977) Biochem. J. 167, 147-154.

Raziuddin, S. (1980) Infect. Immun. 27, 211-215.

Raziuddin, S. and Kawasaki, T. (1976) Biochim. Biophys. Acta 431, 116-126.

Redmond, J.W. (1975) FEBS Lett. 50, 147-149.

Redmond, J.W. (1978) Biochim. Biophys.Acta 542, 378-384.

Redmond, J.W. (1979) Biochim.Biophys.Acta 584, 346-352.

Richards, F.F., Konigsberg, W.H., Rosentron, R.W. and Vorger, J.M. (1975) Science 187, 130-137.

Rietschel, E.Th., Galanos, C. and LUderitz, O. (1975) In Microbiology Ed. Schlessing, D. pp307. American Society for Microbiology, Washington.D.C.

Robins-Browne, R.M., Young, C.R., Levine, M.M. and Craig, J.P. (1980) Infect. Immun. 27, 497-500.

Rodwell, J.D., Gearhardt, P.J. and Karush, F. (1983) J. Immunol. 130, 313.

Rosseaux, J., Picque, M.T. Bazin, H. and Bisterte, G. (1981) Mol. Immunol. 18, 639.

Rowley, D. (1968) Infect. Immun. 29, 278-280.

Sakazaki, R. and Tamura, K. (1971) J. Med. Sci. Biol. 24, 93-100.

Samadi, A.R., Huq, M.I., Shahid, N., Khan, M.U., Eusof, A., Rahman, A.S.M.M., Yunus, M. and Faruque, A.S.G. (1983) Lancet 1, 805-807

Sandulache, R. and Marx, A. (1978) Ann. Microbiol. 127B, 425-435.

Sansano, M.Jr., Reynard, A.M. and Cunningham, R.K. (1985) Infect. Immun. 48, 759-762.

Schalch, W., Wright, J.K., Rodky, L.S. and Braun, D.G. (1979) J. Exp. Med. 149, 923-937.

Schiller,N.L. Hackley, D.R. and Morrison, A. (1984) Curr. Microbiol. 10, 185-190.

Schrank, G.D. and Verwey, W.F. (1976) Infect. Immun. 13, 195-203

Schwaber, J. and Cohen, E.P. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 2203-2207.

Sciortino, C.V., Yang, Z. and Finkelstein, R.A. (1985 9 Infect. Immun. 49, 122-131.

Sears, S.D., Richardson, K., Young, C., Parker, C.D. and Levine, M.M. (1984) Infect. Immun. 44, 439-444.

Sen, A.K., Mukherjee, A.K., Guhathakurta, B., Dutta, A. and Sasmal, D. (1980) Carb. Res. 86, 113-121.

Sen, A.K., Mukherjee, A.K., Guhathakurta, B., Dutta, A. and Sasmal, D. (1979) Carb. Res. 72, 191-199.

Shands, J.W.,Jr., and Chun, P.W. (1980) J. Biol. Chem. 255, 1221-1226.

Shimizu, A. and Honjo, T. (1984) Cell 36, 801-803.

Sidberry, H., Kaufman, B. Wright, D.C. and Sadaff, J. (1985) J. Immunol. Meth. 76, 299-305.

Sincovirs, J.G., Shirato, E. Gyroky, F. Cabiness, J.R. and

Howe, C.D. (1970) In Leukemia Lymphoma, Yearbook Med.Publ., Chicago.

Spira, W.M., Khan, M.U., Saeed, Y.A. and Sattar, M.A. (1980) Bull. W. H. O. 58, 731-740.

Springer, G.F. (1971) Progr. Allergy 15, 9-77.

Stahli, C., Staehelin, T., Miggiano, V., Schimdt,J. and Haring,P. (1980) J. Immunol. Meth. 32, 297.

Stevenson, G., Leavesley,D.I., Lagnado, C.A., Henzenroeder, M.W. and Manning, P.A. (1985) Eur. J. Biochem. 148, 385-390.

Sturm, S., Fortnagel, P. and Timuis, K.N. (1984) Arch. Microbiol. 140, 198.

Talmage, D.W. (1959) Science 129, 1643.

Tempest, D.W. and Ellwood, D.C. (1969) Biotech. Bioengg. 11, 775-783.

Teng, N.N.H., Kaplan, H.S., Hebert, J.M., Moore, C., Douglas, H.W.A. and Braude, A.I. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 1790-1794.

Tortorello, M.L. and Delwiche, E.A. (1984) Curr. Microbiol. 11, 107-112.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4350.

Tsai, C. and Frasch, C.E. (1982) Anal. Biochem. 119, 115-119.

Tsang, J.C., Wang, C.S. and Alanpovic, P. (1974) J. Bacteriol. 117, 786-795.

Verwey, W,F., Watanabe, Y., Phillips, P.E. and Williams, H.R.,Jr. (1965) in Proceedings of the Cholera Research Symposium, Honolulu, Hawaii, U.S. Public Health Service Publication No. 1328.

Vitetta, E., Brooks, K., Chen, Y.W., Isakson, P, Jones, S., Layton, J., Mishra, G., Pure, E., Weiss, E., Word, C., Yuan, D., Tucker, P., Uhr, J. and Krammer, P. (1984) Immunol.Rev. 78, 37.

Wasserman, E. and Levine, L. (1961) J. Immunol. 87, 290-295.

Watanabe, Y. and Verwey, W.F. (1965) Bull. W. H. O. 32, 809.

Watanabe, Y., Verwey, W.F. and Macdonald, E.M. (1965) Bull. W. H. O. 32, 823.

Ways, J.P. and Parham, P. (1983) Biochem. J. 216, 423-432.

Westphal,O. and Jann, K. (1965) Methods in carbohydrate chemistry vol. 5, pp 3-91, Academic press Inc., N.Y.

Wilson M.E. and Morrison, D.C. (1982) Eur. J. Biochem. 128, 137-141.

Woodward, W.E. (1971) J. Infect. Dis. 123, 61-65.

Yuan, D. (1984) J. Immunol. 132, 1566-1570.

Yuan, D. Tucker, P. (1982) J. Exp.Med. 156, 962-974.