POLYMERIC IgA ANTIBODY IN HUMANS AFTER VACCINATION AND IN DISEASE

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ABSTRACT

This thesis examines the relationship between antigen specific polymeric IgA (pIgA) antibody in serum and intestinal IgA antibody in humans.

The difficulties in assessing the human intestinal immune response, the requirement for a simpler method of indirectly assessing intestinal immunity and current knowledge regarding the origins of intestinal and serum IgA in humans are reviewed.

The development and validation of secretory component (SC) binding assays for antigen specific pIgA and total pIgA in human serum are described.

The relationship betwen pIgA antibody responses in serum and the IgA response in intestinal fluid was examined in normal subjects subject to oral vaccination with the live attenuated typhoid vaccine, Salmonella Ty21a and conventional parenteral typhoid vaccination. The serum pIgA response to oral typhoid vaccination and its relationship to the intestinal response was also examined in patients with alcoholic liver disease. In addition, antigen specific serum pIgA was examined in patients with a variety of disorders by looking for serum pIgA to gliadin in patients with Coeliac disease, to Escherichia coli lipopolysaccharide (E.coli LPS) in patients with alcoholic liver disease and Crohn's disease as well as to Campylobacter antigens in patients with Campylobacter enteritis. The immune response of a small sample of unselected patients with IgA nephropathy to oral typhoid vaccination was also investigated.

In normal subjects a short lived pIgA response to both oral and parenteral vaccination was detected whereas an intestinal immune response was only generated by delivery of an antigenic stimulus to the intestine. Levels of typhoid LPS-specific pIgA antibody related to total serum IgA antibody to typhoid LPS rather than intestinal anti-typhoid antibody.

Antigen specific serum pIgA was also detected in the patients with alcoholic liver disease but again there was no relationship to the levels of intestinal antibody. The SC binding assays were also successfully used to measure pIgA antibody in the other patient groups.

The main conclusion of the studies described in this thesis are that while a serum pIgA antibody response can be generated by delivery of an antigenic stimulus to the intestine there is no direct relationship between the level of the serum pIgA antibody and the antibody in the intestine. The regulation of serum pIgA and intestinal IgA appear to be independent.

DECLARATION

I declare this thesis to be based on original data obtained while I was enrolled as an M.D. candidate in the Department of Medicine of the University of Adelaide.

The contents of this thesis have not been previously submitted for a degree in any University and to the best of my knowledge contain no material previously published or written by another person except where due references is made in the text.

Three papers have been published from the work described in this thesis.

Journal of Gastroenterology and Hepatology (1986);1:61.

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Immunology (accepted for publication).

Some of the material has been published in abstract form.

Australian and New Zealand Journal of Medicine (1986);16:p602.

I also give my consent for this thesis to be made available for photocopying and loan.

R.C.A. Bartholomeusz
November, 1989.

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PREFACE

The material in this thesis is presented in nine Chapters. The salient points in the discovery of the secretory immune system, a brief review of its organisation and the difficulties encountered in measuring intestinal immune responses are reviewed in Chapter 1. The reasons for focussing on pIgA in serum and the background to the development of the SC binding assay are also discussed. Chapter 2 contains a description of the material and methods used in the subsequent studies. The development and validation of the assay for antigen specific polymeric IgA is presented in Chapter 3 while Chapter 4 describes measures taken in an attempt to improve the assay. These included the investigation of alternatives to the radioimmunoassay. The development and validation of the assay for total serum polymeric IgA is detailed in the fifth chapter.

Chapter 6 contains a description of studies carried out in normal subjects which examined the immune response to typhoid vaccination. The studies in patients with alcoholic liver disease are presented in Chapter 7. The studies performed in patients with Coeliac disease, Crohn's disease, Campylobacter enteritis and IgA nephropathy are presented in Chapter 8. Chapter 9 contains the final discussion. Supporting figures and legends to the figures are presented at the end of each of the relevant chapters.

CHAPTER 1

INTRODUCTION

I. HISTORY OF THE DISCOVERY OF THE SECRETORY IMMUNE SYSTEM

The mucosal surface of the gastrointestinal tract represents an extensive area that must be defended against potentially harmful micro organisms, chemicals and foreign antigens. A complex secretory immune system has evolved to perform this function. This mucosal immune system has antigen presenting systems, cell types and regulatory mechanisms which are different to those in the systemic immune system (Underdown and Schiff, 1986).

The existence of this protective local immune system was first proposed by Besredka in 1919 when he demonstrated that oral immunisation of rabbits with killed Shiga Bacillus provided protection against fatal dysentery regardless of the level of serum antibody. He postulated that local immunity could be established independently of systemic immunity, and that this was of importance in the resistance of organisms to infections originating in the intestinal tract.

Davies in 1922 showed that specific antibodies could be detected in the stools of patients with

bacillary dysentery several days before such antibodies became evident in serum. Then in the late 1940's Burrows and his colleagues (Burrows, Elliot and Havens, 1947; Burrows and Havens, 1948) in a series of experiments investigating the effect of cholera vaccine in guinea pigs demonstrated a correlation between local faecal antibody (copro-antibody) and protection against experimental infection. They were unable to find a good correlation between serum antibody titres and resistance to oral infection. The copro-antibody titres appeared to be independent of serum antibody titres and were therefore thought not to be derived from serum antibody by simple transudation. Studies in irradiated animals, further highlighted the independence of serum and faecal antibody, by demonstrating that faecal antibody responses to cholera vaccine were inhibited whereas serum antibody levels remained unchanged following appropriate irradiation (Burrows et al, 1950). Subsequent studies by Freter et al (1956) using a model of acute fatal cholera infection in guinea pigs, also showed that copro-antibody and not serum antibody was protective. Copro-antibody was also found to be protective in experimental cholera in rabbits (Jenkin and Rowley, 1960). Then in 1962, Freter showed that oral immunisation was effective in generating copro-antibody in humans. He subsequently demonstrated that

antibody could be detected in duodenal fluid after oral immunisation (Freter and Gangarosa, 1963).

These studies led to the realisation that in certain enteric infections circulating serum antibody had relatively little significance, and was only indirectly related to resistance to such infections. Local immunity and local antibodies were believed to be of primary importance in defence against infection at mucosal surfaces.

Then came the important discovery of the IgA class of antibody (Heremans et al, 1959). Hanson et al (1961) showed that it predominated in breast milk and had unique characteristics. It was soon realised that IgA was the principal class of immunoglobulin in external secretions (Chodirker and Tomasi, 1963). This secretory IgA was shown to have unique physiochemical and immunochemical properties because of its dimeric nature and association with a glycoprotein, secretory component (Tomasi et al, 1965). These discoveries together with the finding of a predominance of IgA secreting plasma cells in glands and mucous membranes facing the external environment formed the basis for the suggestion that there existed a more or less distinct immunological system characteristic of external secretions (Tomasi et al,

1965). The evidence for the existence of this secretory immune system was presented in a review by Tomasi and Bienenstock (Tomasi and Bienenstock, 1968).

After the identification of secretory IgA as the dominant immunoglobulin in external secretions, others went on to localise the site of antibody production by showing that there was a marked predominance of IgA containing cells in the lamina propria of the intestine (Crabbé and Heremans, 1966). It was also shown that if experimental animals were fed antigens, specific IgA forming cells appeared in the lamina propria of the gut and in the mesenteric lymph nodes (Crabbé et al, 1969). Crabbé and colleagues (Crabbé et al, 1970) suggested that the Peyer's patches of the small intestine were the primary source of lamina propria IgA producing cells. This was confirmed by Craig and Cebra (1971) who showed that lymphoblasts from Peyer's patches were a richer source of IgA precursors than the spleen, lymph nodes or blood lymphocytes and that these lymphoblasts from the Peyer's patches could re-populate the lamina propria of irradiated rabbits with IgA plasma cells.

Several studies have shown that the presentation of an immunogen to the luminal surface of the intestine leads to the appearance of antigen specific

IgA antibody cells in the intestinal lamina propria (Pierce and Gowans, 1975; Pierce et al, 1978; Husband and Gowans, 1978). After oral immunisation specific IgA antibodies appear in gut secretions (Bienenstock, 1974). These specific IgA antibodies may also appear in mucosal secretions distant from the gut such as in the bronchial tract (Montgomery et al, 1978) and salivary, lacrimal and mammary glands (Montgomery et al, 1978; Mestecky et al, 1978; Ahlstedt et al, 1977; Montgomery et al, 1974). These observations supported Bienenstock's suggestion of a common mucosal immune system (Bienenstock, 1974).

II. REVIEW OF THE ORGANISATION OF THE INTESTINAL IMMUNE SYSTEM AND PRODUCTION OF INTESTINAL IgA ANTIBODY

In recent years there have been numerous reviews published concerning the intestinal immune system and immunoglobulin A (Tomasi and Bienenstock, 1968; Heremans, 1974a; Bienenstock, 1974; Lamm, 1976; Tomasi, 1976; Parrott, 1976a&b; Walker and Isselbacher, 1977; Bienenstock and Befus, 1980; Bienenstock and Befus, 1983; Brandtzaeg et al, 1985; Underdown and Schiff, 1986). A brief review of the intestinal immune system with the emphasis on humoral immunity is presented here.

The gastrointestinal tract has a complex highly organised immune system. There are diffusely scattered infiltrates of lymphoid cells throughout the lamina propria and in between epithelial cells, and specialised aggregates of lymphoid tissue known as the gut associated lymphoid tissue (GALT). In humans these focal aggregates of tissue are present in the tonsils, appendix and Peyer's patches which are the principal sites of interaction between the cells of the immune system and antigens in the lumen.

The Peyer's patches are covered by a specialised epithelial cell, the M cell (Owen and Jones, 1974) which is capable of transporting antigens into the lymphoid follicles in the Peyer's patches (Owen, 1977). There are aggregates of B and T lymphocytes under this epithelium (Parrott, 1976a&b). Following antigen stimulation antigen specific IgA antibody appears in intestinal secretions (Bienenstock, 1974) and antigen specific IgA containing cells appears in the lamina propria (Pierce and Gowans, 1975; Pierce et al, 1978; Husband and Gowans, 1978). Crabbé et al (1970) suggested that the Peyer's patches were the source of the lamina propria IgA cells. It is now known that the precursors of the IgA cells leave the Peyer's patches and migrate in lymph through the mesenteric nodes and enter the circulation via the

thoracic duct. They then migrate to secretory sites (Parrott, 1976a).

In the lamina propria the IgA secreting cells are the predominant plasma cells (Crabbé et al, 1965). These IgA cells produce polymeric (dimeric) IgA (Brandtzaeg, 1973). The polymeric IgA containing J chain binds to secretory component on the epithelial cell membrane (Crago et al, 1978; Brandtzaeg, 1978), is endocytosed and transported across the cell to be released into the lumen (Brown et al, 1976; Nagura et al, 1979), covalently bound to dimeric IgA as secretory IgA.

Secretory IgA is an 11s dimer of M.W. 390,000 composed of two 7s IgA monomers, a joining peptide the J chain (M.W. 15,000), and a glycoprotein secretory component (M.W. 75 to 80,000). The J chain first identified by Halpern and Koshland (1970), links the two IgA monomers before secretion from the plasma cell (Koshland, 1975).

Secretory component (produced by epithelial cells) is a glycoprotein which acts as the receptor for dimeric IgA (Crago et al, 1978). The J chain and secretory component represents the "lock and key" in the translocation of dimeric IgA into the lumen

(Brandtzaeg and Prydz, 1984).

Secretory component also renders secretory IgA resistant to proteolysis by intestinal enzymes (Tomasi and Bienenstock, 1968). In addition to being the predominant immunoglobulin in intestinal secretions, secretory IgA predominates in the external excretions at other secretory sites such as the salivary glands, bronchus and breast (Tomasi et al, 1965).

The functional properties of secretory IgA have been reviewed recently (Underdown and Schiff, 1986). Secretory IgA has been shown to prevent the adherence of bacteria to mucosal surfaces (Williams and Gibbons, 1972; Fubara and Freter, 1973; Svanborg-Eden and Svennerholm, 1978) and prevent the uptake of luminal dietary protein antigens (Walker and Isselbacher, 1974). It also helps to neutralise toxins and infective organisms (Pierce and Sack, 1977; Tagliabue et al, 1983).

It may have a role in antibody dependent cell mediated cytotoxicity (Underdown and Schiff, 1986). It is unable to bind complement efficiently and this may be an advantage in the intestine where the initiation of an inflammatory reaction may damage the integrity of the mucosal surface. The prime function

of the intestinal IgA is to protect the host from invading microorganisms and foreign chemicals and antigens. The mucosal IgA system has been described as a "strategic defence initiative" designed to prevent invading microorganisms gaining a foothold on the mucosal wall (Underdown and Schiff, 1986).

III. THE ASSESSMENT OF INTESTINAL IMMUNITY IN HUMANS

In view of the dissociation that often occurs between the secretory and serum antibody response to antigens at secretory surfaces, the assessment of intestinal immunity through the measurement of serum antibody is of little value. In order to measure the local immune response it has been necessary to measure the antibody response in intestinal secretions. There have been several studies which have examined the secretory antibody response to infection and oral immunisation in humans.

An antibody response in intestinal secretions has been demonstrated following acute cholera infection (Freter et al, 1965; Northrup and Hossain, 1970; Waldman et al, 1972). Intestinal antibody has been demonstrated following several other enteric infections such as Escherichia coli gastroenteritis, (McNeish et al, 1975), Shigellosis (Reed and Williams, 1971), salmonella and shigella gastroenteritis

(LaBrooy et al, 1980), typhoid fever (Chau et al, 1981), and rota virus (Davidson et al, 1983) infection.

Intestinal antibodies have also been examined in diseases of the gastrointestinal system such as coeliac disease (Katz et al, 1968; Ferguson and Carswell, 1972; LaBrooy et al, 1986).

As mucosal IgA antibodies are largely derived from local synthesis and not from the circulation parenteral immunisation may not be effective in stimulating the production of intestinal antibody. For instance oral immunisation with the live attenuated polio vaccine (Sabin) led to an intestinal IgA antibody response while the parenteral (Salk) vaccine did not (Ogra et al, 1968). Therefore attempts have been made to induce the production of local antibody by oral immunisation. The possibility of oral immunisation against enteric infections has been explored. Oral vaccines against typhoid (Germanier and Fürer, 1975), Shigella (Formal et al, 1981), and cholera (Svennerholm et al, 1984) have been evaluated.

The generation of intestinal antibody by these vaccines has been studied. The mucosal immune response to immunisation with the cholera B subunit and with

combined B subunit whole cell cholera vaccines has been evaluated (Svennerholm et al, 1982; Svennerholm et al, 1984; Jertborn et al, 1984). As oral administration of antigens induces the appearance of secretory antibodies at other mucosal surfaces the induction of a generalised secretory IgA response in external secretions by oral immunisation to prevent various systemic infections is being explored (Mestecky, 1987).

IV. DIFFICULTIES IN STUDYING INTESTINAL IMMUNITY IN HUMANS

1. INTESTINAL ANTIBODY

The assessment of the intestinal immune response through the detection of intestinal antibodies is beset with problems. Although initial interest in intestinal antibody arose through studies in which faecal or copro-antibody was measured the results obtained in attempting to measure antibody in stool have been inconsistent. The reasons for this have been reviewed by Freter (Freter, 1962), who demonstrated that there was breakdown of the antibody in stools. Attempts have been made to reduce the breakdown of antibody by purgation. The "Lavage method" described by Sack et al (1980) in which subjects drink 250ml of an isotonic solution every ten minutes until watery diarrhoea ensues and 1000ml of a

watery stool is obtained has been used to demonstrate an intestinal antibody response after cholera disease and after immunisation with cholera vaccines (Svennerholm et al, 1982; Svennerholm et al, 1984; Jertborn et al, 1984). An intestinal antibody response to naturally acquired enterotoxigenic Escherichia coli disease has been demonstrated using this method (Stoll et al, 1986). However, this method is time consuming and requires motivated cooperative subjects and good laboratory facilities which are not always available in the field.

The other reliable method of studying intestinal antibody has involved intestinal intubation. This too is a laborious process which demands the co-operation of the subject and some skill on the part of the investigator. Gastric aspirate is not a good source of antibody as the immunoglobulins are degraded by gastric acid (McLelland et al, 1971), and pepsin (Sampson et al, 1973). The intestinal tube has to be positioned in the duodenum or jejunum in order to obtain suitable fluid. Fluoroscopy has to be used to position the tube thus exposing the subject to radiation.

During collection and storage intestinal antibodies are exposed to proteolytic enzymes, such as

trypsin and chymotrypsin. Fortunately secretory IgA is resistant to digestion by trypsin (Brown et al, 1970). Various attempts have been made to reduce the enzyme damage. These have included heating to 56^{o}C for an hour to denature the enzymes (Plaut and Keonil, 1969), and the addition of trypsin inhibitors, such as soya bean trypsin inhibitor (Waldman et al, 1971), and Trasylol (Sampson et al, 1973). The rapid freezing of intestinal samples proved to be just as effective (Sampson et al, 1973; Horsfall and Rowley, 1979). Despite these problems sensitive and reproducible radioimmunoassays and enzyme linked immunosorbent assays are now available, and the intestinal antibody response to infection and immunisation can be measured.

Although the measurement of intestinal antibody remains the most reliable method of determining the intestinal immune response intestinal intubation and the lavage method are inconvenient and are not suitable for the assessment of the immune response to vaccines administered in field trials.

2. OTHER MEASURES OF INTESTINAL IMMUNITY

To overcome the difficulties in obtaining and measuring intestinal antibody the use of other body fluids such as saliva, breast milk and serum for

assessing intestinal immunity indirectly have been explored.

In view of the concept of IgA secretory cells homing to secretory sites (Tomasi, 1976), and the concept of a common mucosal immune system (Bienenstock, 1974), other fluids that have secretory IgA as the predominant immunoglobulin have been examined in studies of the local intestinal immune response. Specific antibody to salmonella typhimurium was detected in the colostrum of women infected 3 months before term (Allardyce et al, 1974), and oral administration of non-pathogenic strains of E.coli led to the appearance of cells secreting IgA antibodies to the bacterium in colostrum (Goldblum et al, 1975).

The infrequent availability of colostrum limits its usefulness. Saliva is obtained more easily and Mestecky et al (1978) demonstrated an antibody response in parotid saliva after oral immunisation with a strain of streptococcus mutans (Mestecky et al, 1978). However, the use of saliva for assessing the intestinal immune response has not been a universal success. For instance, salivary antibodies could not be detected after salmonella or shigella gastroenteritis, despite the presence of intestinal antibodies (LaBrooy et al, 1980). More recently an

antibody response was induced in saliva in Swedish volunteers given an oral cholera vaccine (Jertborn et al, 1984) and a variable antibody response in both saliva and breast milk was demonstrated in Bangladeshis' after both cholera disease and after immunisation with the combined B sub unit-whole cell vaccine (Svennerholm et al, 1984). However, Jertborn et al, 1986) found that extra intestinal antibody in saliva and breast milk did not directly reflect intestinal immunity after cholera vaccination or natural disease. The relationship between antibody in saliva and breast milk to intestinal antibody after enterotoxigenic E.coli disease was also not clear cut (Stoll et al, 1986).

The measurement of serum antibody even in the IgA class has been of limited value because of the dissociation between the immune response in serum and secretions. Even when the IgA class antibodies are examined there is no correlation between the intestinal antibody response and serum antibody (LaBrooy et al, 1980; Chau et al, 1981; Jertborn et al, 1986). The lack of correlation between total serum antibody or serum IgA antibody with intestinal IgA antibody after infection or oral immunisation in humans contrasts with studies in some animal species in which oral immunisation generating a local IgA

response in the intestine is frequently accompanied by a marked IgA antibody response in serum (Heremans, 1974a). For instance, in mice serum IgA antibody responses have appeared to parallel intestinal antibody responses (Hohmann et al, 1979). In these animals the intestinal lymphoid tissue makes a significant contribution to serum IgA and most of the IgA in serum is polymeric (Heremans, 1974a; Lamm, 1976), whereas in humans only 10-15% of the serum IgA is polymeric (Heremans, 1974b), which is produced in the bone marrow (Hijmans et al, 1971).

The failure to demonstrate a correlation between serum and intestinal IgA antibody in humans may be due to the failure to focus on the polymeric fraction of serum IgA. The aim of this thesis was to determine if the antibody in the polymeric fraction of serum IgA would provide a correlate of intestinal antibody after oral immunisation or enteric infections. The reasons for focussing on serum polymeric IgA (pIgA) and current knowledge on the origins of serum pIgA in humans are reviewed in the next section.

V. SERUM POLYMERIC IgA

Local production of pIgA is the basis for secretory immunity. The intestine and salivary glands contain cells that preferentially produce pIgA containing J

chain (Brandtzaeg, 1973; Kutteh et al, 1982a). This pIgA binds to a glycoprotein secretory component (SC) on the basolateral surface of the intestinal epithelial cells and is translocated into the lumen covalently bonded to SC as secretory IgA (Brandtzaeg, 1974; Brown et al, 1976; Brandtzaeg, 1978; Crago et al, 1978; Nagura et al, 1979).

IgA producing cells have been located in all lymphoid tissues but the intestine is the major source of serum IgA in several animal species such as dogs and rats (Heremans, 1974a; Lamm, 1976). In the rat serum IgA is equally divided between monomeric and polymeric forms and most of the serum pIgA is derived from plasma cells in the lamina propria of the intestine (Vaermans et al, 1973). In the rat the mesenteric lymph which flows into the thoracic duct and enters the circulation is an enriched source of pIgA (Manning et al, 1984). In dogs too most of the serum IgA is pIgA and once again the mesenteric lymph is an enriched source of pIgA (Vaermans and Heremans, 1970). In mice, the decrease in serum IgA that follows whole body irradiation can be prevented by shielding the intestine (Bazin et al, 1970) suggesting that the intestine is a major source of serum IgA. In many of these animals the delivery of an antigenic stimulus to the intestine by oral immunisation

generates a specific plasma cell response in the gut associated lymphoid tissue which is IgA in character and associated with an antigen specific IgA antibody response in serum (Crabbé et al, 1969; Dolezol and Bienenstock, 1971).

However, the situation is different in humans. Human thoracic duct lymph is not enriched for IgA. It contains approximately the same concentration of IgA as in serum, with the same distribution of monomeric and pIgA as serum (Kaartinen et al, 1978; Brown et al, 1982).

In humans, IgA constitutes a significant proportion of the total serum immunoglobulin (about 6 to 15%) and is present in relatively high concentrations of 1 to 2mg per ml (Conley and Delacroix, 1987). There are 3 distinct forms of IgA in human serum; IgA monomers, IgA polymers (mainly dimers) composed of 2 or more disulphide linked IgA monomers and J chain, and thirdly, small amounts of secretory IgA, a polymeric molecule composed of 2 to 4 disulphide bond linked monomers, J chain and secretory component.

In humans approximately 85% of serum IgA is monomeric IgA, 10-15% pIgA and 1% secretory IgA

(Heremans, 1974b). This contrasts with the IgA in secretions which is predominantly polymeric. There are 2 subclasses of IgA in human serum, IgA1 and IgA2. IgA1 constitutes 85% of serum IgA (Delacroix et al, 1982a; Delacroix et al, 1983). These 2 subclasses are present in equal amounts in secretions (Delacroix et al, 1982a).

Heremans (Heremans, 1974a) proposed that each form of IgA had its own tissue of origin. Human bone marrow is believed to be the major source of serum immunoglobulin (Benner et al, 1981) and the main source of circulating IgA (Hijmans et al, 1971). Bone marrow cells produce predominantly monomeric IgA (Radl et al, 1974), while cells in the intestine and salivary gland preferentially produce pIgA (Brandtzaeg, 1973; Kutteh et al, 1982a).

VI. THE SOURCE OF SERUM pIgA IN HUMANS

Heremans (1974a) and Lamm (1976) suggested that the pIgA in serum originated from mucosal sites. Bull et al (1971) in perfusion studies on segments of human intestine showed that human intestine could synthesise IgA which was polymeric and that most of the IgA in the venous effluent was pIgA. Indirect evidence for the intestinal origin of pIgA was obtained by Brandtzaeg (1973) and Radl et al (1974), who showed

that SC bound to a higher number of intestinal lamina propria IgA plasma cells than bone marrow cells.

Then Kutteh et al (1982a) examined the amount and molecular forms of IgA secreted in vitro by cells obtained from human spleen, tonsils, lymph nodes, intestinal mucosa, bone marrow and peripheral blood. Cells were examined for the presence of cytoplasmic immunoglobulin and J chain, and for the amounts and molecular forms of intra-cellular and secreted IgA. The presence of J chain in the cytoplasm was correlated with the potential to secrete pIgA.

Cultured bone marrow cells were the largest producers of total IgA and approximately 90% of this was monomeric. Spleen cells also produced predominantly monomeric IgA. Human tonsil and lymph node cells secreted approximately equal amounts of monomeric and pIgA. Intestinal mucosal cells produced predominantly pIgA and cytoplasmic J chain was found in 89% of IgA containing cells from the mucosa.

It is possible however, that tissues other than those at secretory sites contribute to the pIgA in serum. In the study of Kutteh et al (1982a) up to 40% of the pIgA secreted by cultured lymph node cells was in the polymeric form. In another study the IgA

produced in vitro by peripheral blood lymphoctes stimulated with various mitogens such as pokeweed mitogen, Escherichia coli lipopolysaccharide or Epstein Barr virus was predominantly pIgA (Kutteh et al, 1980). However, it was suggested that these pIgA producing cells in peripheral blood were precursors of IgA producing cells originating from the intestine with the potential to repopulate mucosal tissue (Kutteh et al, 1980).

The controversy regarding the source of pIgA in human serum continues. André et al (1980) suggested that the pIgA and IgA2 in human serum was derived from mucosal surfaces. The presence of pIgA and IgA2 in the renal mesangium of patients with IgA nephropathy led them to suggest a mucosal origin of the renal deposits (André et al, 1980). Unsworth et al (1982) detected dimeric IgA containing J chain, capable of binding SC in skin deposits of patients with dermatitis herpetiformis, a gluten sensitive disease where treatment with a strict gluten-free diet leads to both remission of the skin and intestinal lesions. They suggested that the dimeric IgA in the skin deposits had originated from the gut associated lymphoid tissue.

Therefore despite some uncertainty regarding the

origin of serum pIgA in humans there was evidence to suggest that it may originate from the intestine. If this suggestion was correct it was possible that the measurement of antigen specific pIgA antibody in serum could reflect the intestinal antibody response to an enteral antigenic stimulus. The aim of the studies performed and described in this thesis was to determine whether such a relationship between serum pIgA antibody and intestinal antibody existed. In order to examine the hypothesis it was essential that antigen specific pIgA antibody in serum could be readily measured. When the studies commenced no assays for the measurement of antigen specific pIgA antibody had been described. The development of a suitable assay for this purpose became a prerequisite for the studies. Assays for the measurement of total but not antigen specific pIgA in serum were available. These methods of measuring total pIgA in human sera are described below.

VII. THE MEASUREMENT OF pIgA IN HUMAN SERA

The measurement of pIgA in human serum has always presented difficulties. The methods used have with the exception of the method described by Newkirk et al (1983) involved separation of IgA into polymeric (>9S) and monomeric (7S) fractions by velocity sedimentation in either sucrose density gradients or by gel

chromatography (Lopez Trascasa et al, 1980; Sancho et al, 1982; Kutteh et al, 1982a&b; Delacroix et al, 1983).

These approaches are laborious, time consuming and result in loss of material during fractionation. Some of these assays did not distinguish between biosynthetically produced pIgA and aggregates of IgA which may have formed during storage. Some did not distinguish between secretory IgA and pIgA.

Lopez Trascasa et al (1980) using gradient centrifugation found an increased proportion of serum in the 9 to 21s fraction in patients with IgA glomerulonephritis. 5 to 40% sucrose density gradients were utilised in this study. The sucrose was dissolved in 0.15M Tris HCl pH 7.4 or in 0.15M Glycine HCl pH 2.8. 50ul of serum diluted 1 in 10 was centrifuged at $4^{o}C$ for 16 hours at 170,000g and 200ul fractions collected. The IgA in each fraction was measured by R.I.A. Acid treatment reduced the proportion of 9 to 21s IgA. A significant decrease in the 13 to 21s fraction with acid conditions suggested that they were immune complexes. J chain was detected in the fractions of high molecular weight and an affinity of SC for the IgA in these fractions was also demonstrated.

Sancho et al (1982b) utilized sucrose density gradients of 5 to 40% and sucrose in 0.15M Tris HCl pH 7.4 or 0.15M Glycine HCl pH 2.8. 50ul serum samples diluted 1 in 10 were analysed. Using this method monomeric and pIgA containing immune complexes and pIgA was detected in sera from patients with alcohol induced liver disease. All fractions between 9 to 21s decreased at acid pH suggesting that immune complexes were present. Even after centrifugation at pH 2.8 the patients had high levels of IgA in the 9 to 13s fractions suggesting the presence of true pIgA.

Kutteh et al (1982) used gel chromatography to analyse the quantity and molecular forms of IgA in sera from patients with liver disorders. Samples were fractionated on a 1.6 x 90cm column of Ultrogel ACA 22. 25 to 45% of the IgA was represented by polymeric forms in patients with cirrhosis whereas less than 8% of the IgA was polymeric in normal sera. About 5% of the pIgA was actually secretory IgA. This was demonstrated by precipitation with anti secretory component antibody. The presence of J chain in the high molecular weight fractions was also demonstrated.

Sucrose density gradient ultra centrifugation was utilised by Delacroix et al (1983). Sera were divided

into 31 fractions of 0.4ml after sucrose density gradient ultra centrifugation for 16 hours at 38,000 R.P.M. on 5 to 21% gradients. The fractions were analysed by immunoassay. The buffers used were phosphate buffered saline pH 7.4 or 0.1M Glycine HCl buffered saline pH 3.0. Sucrose density gradient ultracentrifugation at pH 3.0 was only performed in patients with greater than 20% pIgA to shift pIgA immune complexes to a less dense region of the gradient. The presence of J chain in the high molecular weight fractions was demonstrated by immuno electrophoresis using rabbit anti J chain antibodies.

A different approach was adopted by Newkirk et al (1983) who described a SC binding assay for the estimation of pIgA in serum. SC was radiolabelled using the iodine monochloride or iodogen method. The labelled SC was incubated with pIgA for 10 to 18 hours at $4^{o}C$ and this was followed by the addition of an excess of anti IgA for 18 hours at $4^{o}C$ which resulted in the maximum precipitation of the pIgA-SC complexes. Maximum binding of SC to pIgA was 70 to 85% of the counts added. The SC did not bind IgG or monomeric IgA (free or heat aggregated). It did bind IgM and the presence of greater than 2ug IgM per 10ul interfered with the assay. IgM in those sera were removed with glutaraldehyde polymerised anti IgM

antibodies. This assay was claimed to have a sensitivity of 0.05ug per 10ul.

The authors emphasised that the SC binding assay distinguished between serum pIgA and secretory IgA or aggregated IgA and avoided the extensive manipulation of sera required for sucrose density gradient centrifugation or gel filtration.

Using the methods described investigators have attempted to examine the contribution of pIgA to serum IgA in health and disease.

Heremans (1974b) estimated that normal serum contained 10 to 15% (0.2 to 0.3mg per ml) of polymeric (chiefly dimeric) IgA. Kutteh et al (1982b) found that less than 8% of serum IgA was polymeric in normal controls. According to Delacroix et al (1983) in healthy adults pIgA contributes 4 to 22% (mean 12%) of the total serum IgA. Using a SC binding assay the quantity of pIgA found in sera from 30 normal subjects was 0.13 ± 0.08mg per ml (±1 S.D.) which constituted 11.3 ± 5.3% (±1 S.D) of the total IgA (Newkirk et al, 1983).

Changes in the proportion of the pIgA fraction of serum IgA have been reported in liver disease (Tomasi

and Grey, 1972; André and André, 1976; Sancho et al, 1982; Kutteh et al, 1982b; Delacroix et al, 1983; Newkirk et al, 1983), IgA nephropathy (André et al, 1980; Lopez Trascasa et al, 1980; Newkirk et al, 1983; Valentjin et al, 1984) and Crohn's disease (Delacroix et al, 1983).

VIII. BACKGROUND TO THE DEVELOPMENT OF THE SECRETORY COMPONENT BINDING ASSAY FOR POLYMERIC IgA ANTIBODY

The studies performed towards this thesis sought to determine the relationship of intestinal antibody to serum pIgA antibody. Rather than utilizing velocity sedimentation in sucrose density gradients or gel chromatography to separate IgA into polymeric and monomeric fractions and then performing immunoassays for antigen specific pIga antibody, a new solid phase assay for detecting pIgA antibody bound to antigens adherent to the wells of microtitre plates was developed which utilised secretory component (SC) to detect the pIgA antibody.

This approach was adopted in view of the known affinity of SC for pIgA. Previous experimental evidence that SC could bind to pIgA in vitro is presented below.

The first report that SC was able to bind to IgA came from Tomasi and Bienenstock (1968) using SC obtained from colostral IgA by reduction alkylation. Hanson et al (1969) showed that native free SC formed complexes with serum IgA. Brandtzaeg (1971) found that the in vitro binding of SC was relatively specific for IgA although there was some evidence of SC IgM association. A low yield of SC IgA complexes were obtained in these studies.

The realisation that SC bound to pIgA and not to monomeric IgA came later. The observation that a particular immunoglobulin configuration was necessary for SC binding came from studies performed with secretory IgA obtained from rabbits. When bound SC was removed from secretory IgA this IgA showed a high affinity for SC (Lawton et al, 1970; O'Daly and Cebra, 1971).

The in vitro combination of free SC with human pIgA was demonstrated by Mach (1970) and independently by Radl et al (1971). Mach isolated SC from human and bovine milk and showed that it combined in vitro with human and mouse pIgA. He showed that it did not bind to IgG or monomeric IgA. Artificially prepared IgA or IgG polymers did not bind SC. Reduction and alkylation experiments suggested that disulphide

bridges were formed in the combination.

Radl et al (1971) showed that SC obtained from mixed saliva and tears of patients with selective IgA deficiency combined with pIgA but not with IgG or IgD. They found that higher polymers of IgM with sedimentation coefficients of 25s to 30s bound SC as well. This in vitro combination of SC with pIgA and IgM was demonstrated using immunoelectrophoresis and immunoselection.

The fact that SC bound preferentially to pIgA and IgM suggested a role for the J chain. Eskeland and Brandtzaeg (1974) reported than an IgM polymer lacking J chain failed to bind SC. They also showed that SC could bind to 19s IgM polymers.

Information on the nature of the binding of SC to pIgA and IgM was obtained by Weicker and Underdown (1975). They showed that both dimeric IgA and pentameric IgM bound ^{125}I labelled SC with approximately one SC binding site per mole of polymers. An apparent association constant of 10^8 M^{-1} was calculated to govern the binding of SC to the polymers. They too obtained evidence that disulphide bonds were formed in the ^{125}I SC-pIgA complex. Complexes of SC and pIgA were presumed to involve

covalent bond formation as they did not dissociate in guanidine HCl. In contrast the SC IgM complex dissociated in denaturing solution suggesting that the SC IgM complex was held together by non covalent bonds. Brandtzaeg (1977) reported that the non covalent forces involved in complex formation were much stronger for IgM than pIgA. The equilibrium constant of association Ka for both polymers was in the range 10^7-10^8 M^{-1} but appeared to be 2.7 to 12.5 times higher for IgM than pIgA. In a competitive binding test SC had a 5 to 30 times better affinity for IgM. Brandtzaeg postulated that this was due to the higher molar content of J chain in IgM which affects the configuration of the SC binding site. There was a greater tendency for disulphide exchange reactions to occur when SC bound pIgA. Oxidation dependent stabilisation and packing of the SC immunoglobulin complex took place more readily for the SC pIgA complex.

These findings were similar to those of Socken and Underdown (1978). They showed that SC bound to IgM with 2 to 5 fold greater affinity than to dimeric IgA and that the SC pIgA binding involved covalent bond formation. Binding to IgM was predominantly non covalent. The number of binding sites in human pIgA was slightly lower than that found in human IgM (0.7

vs 1.1). This has also been reported by Weicker and Underdown (1975) and Brandtzaeg (1977). Lindh and Björk (1974, 1976a,b) have also studied the association of SC with pIgA and IgM. They (Lindh and Björk, 1976a) found evidence that the disulphide bonds between SC and pIgA are formed after non covalent association of the two proteins by a sulphydryl group-disuphide bond exchange reaction in which the free sulphydryl groups in the IgA polymer initiate the reaction by reducing a reactive disulphide bond on SC. This exchange reaction, a so called disulphide interchange reaction leads to the formation of interchange disulphide bonds between SC and pIgA. They confirmed that the binding of SC to IgM was non covalent (Lindh and Björk, 1976b).

In the light of this data an assay where SC could be used as a probe to detect the pIgA fraction of serum that binds to antigen immobilised on a micro titre plate was developed. An assay for total serum pIgA was also developed. The development and application of these assays to the study of the human secretory immune system is presented.

CHAPTER 2

MATERIALS AND METHODS

I. GENERAL METHODS USED TO PREPARE AND ANALYSE THE PROTEINS

1. Gel Chromatography

Sephacryl S200, S300 and Sephadex G75 (Pharmacia) were used for fractionating proteins by gel chromatography. Columns 90 to 100cm long with diameters of 1.5 to 3.5cms were used. The Pharmacia handbook on gel-chromatography was followed in preparing the columns. The gel was swollen in an appropriate buffer at $4^{o}C$ for 72 hours before use. After degassing, it was poured into the column, with precautions to prevent any trapping of air, swirling of beads and uneven layering. Buffer was run through the columns, first downwards and then upwards. The exclusion volume and bed volume of the columns was determined using Dextran blue (excluded by the beads) and p-nitro phenol (totally trapped by beads). The volume of the sample applied to the column was less than 2% of the volume of the column. The column was used with a LKB fraction collector, ultra violet absorption scanner and printer so that the protein content of the fractions were visually displayed and recorded.

2. Ion Exchange Chromatography

Ion exchange chromatography was performed using diethyl amino ethyl (DEAE) cellulose (DE52, Whatman). The gel was prepared according to the manufacturer's instructions. The gel was repeatedly washed in the starting buffer to remove fines and allow the gel to equilibrate with the buffer. When the pH and conductivity of the supernatant left after the wash was the same as the initial buffer, the gel was degassed and poured into a column taking care to avoid swirling and trapping of bubbles. An open system was used with a 5cm head of buffer above the gel at the upper end of the column and using downward flow. The pH and conductivity of the effluent was checked to ensure that it was the same as the initial buffer. The sample was dialysed extensively against the initial buffer and then applied carefully onto the column after removing excess head of buffer. The column was then washed to elute off the unbound protein. Stepwise changes of buffer were used to elute off proteins bound to the column.

The column was connected to an LKB fraction collector and UV absorption scanner and printer so that the protein content of the fractions was visually displayed. The stepwise changes in the eluting buffer were only made after the absorption profile had

reached a steady base line.

3. Affinity Chromatography

Affinity chromatography was performed using columns of protein coupled to commercial Cyanogen bromide activated Sepharose 4B (Pharmacia) according to the instructions issued by the manufacturer. The gel was initially resuspended in 1mM hydrochloric acid (HCl) and then washed with 1mM HCl. The protein to be coupled was dissolved at a concentration of 10mg/ml in the coupling buffer (0.1M sodium bicarbonate 0.5M sodium chloride, pH 8.3). This was mixed with the gel in a test tube and rotated end over end using a rotary stirrer for 2 hours at room temperature. The ratio of gel to protein during this process was 1g of swollen gel to 5ml of the protein solution. Unbound protein was washed away with coupling buffer on a sintered glass funnel. Remaining active sites on the gel were quenched with 1M ethanolamine at pH 8.0 for 2 hours. Three washing cycles to remove non covalently adsorbed protein were used consisting of a wash at pH 4.0 (0.1M acetate buffer with 1M sodium chloride) followed by a wash at pH 8.0 (0.1M borate buffer containing 1M sodium chloride). The effectiveness of the coupling was checked by determining the protein losses in the washing steps. The efficiency of coupling ranged

between 80 to 95%.

On using these columns protein was run on and washed through with 0.1M phosphate buffer pH7.6 (sodium hydrogen phosphate, potassium di hydrogen phosphate Na_2HPO_4, KH_2PO_4). Elution of bound protein was performed with 1 to 3M sodium or potassium thiocyanate in 0.1M phosphate buffer at pH 7.4. The whole procedure was performed at room temperature using an LKB fraction collector, absorption scanner and printer. The eluate was immediately dialysed in the cold against repeated changes of phosphate buffered saline PBS (0.04M phosphate, 0.15M sodium chloride, pH 7.4) and the column washed through with 0.1M phosphate buffer.

4. Analysis Of Protein Preparations

This was done by immunodiffusion, using immunoelectrophoresis and Ouchterlony analysis (Ouchterlony and Nilsson, 1973), enzyme linked immunosorbent assays (ELISA) and by electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate (SDS) after reduction with 2-mercaptoethanol (Laemmli, 1970). Commercial immunoglobulin heavy chain specific, alkaline phosphatase-conjugated antibodies (Kirkegaard Perry Laboratories) were used in the ELISA.

The concentrations of the protein in purified preparation was based on the determination of the optical density of the solutions at a wave length of 280mu using a Zeiss spectro photometer.

The concentrations of the purified proteins were measured using an $E^{1cm}_{1\%}$ at 280nm of 13.4 for IgA (Heremans, 1974a) 13.8 for IgG (Heremans, 1974a) 11.8 for IgM (William and Chase, 1968) and 12.7 for secretory component (Kobayashi, 1971).

5. Standards And Antisera Used In The Analysis Of Protein Preparations

In the Ouchterlony and immunoelectrophoresis analysis of proteins and in the radial immunodiffusion assays commercial heavy chain specific antisera were used (Behring anti α chain, ORCI 14/15; anti γ chain ORCM 14/15; anti μ chain ORCK 14/15). Antisera against secretory component was obtained from the same source (Behring anti SC OTPL 04/05).

The same heavy chain specific antisera were used in the single radial immunodiffusion assays (Mancini et al, 1965). The reference preparation was a commercial polyclonal immunoglobulin (Behring standard human serum ORDT 06/07) except for measuring secretory IgA where a secretory IgA standard was used.

6. ELISA Analysis Of Protein Preparations

The purity of the protein preparations was analysed by ELISA using heavy chain specific antibodies coupled to alkaline phosphatase (Kirkegaard and Perry Laboratories). Polyvinyl microtitre plates with 96 wells (COSTAR Data Packaging Company, Cambridge, Mass, USA) were coated with the protein diluted in coating buffer (0.05M sodium carbonate buffer pH 9.6) incubating 100ul per well at $4^{o}C$ overnight. Serial doubling dilutions of the protein starting from usually 10ug per ml were incubated in duplicate on the plate. The plates were blocked with 0.5% bovine serum albumin (BSA) in phosphate buffered saline (.04M phosphate, .15M sodium chloride pH 7.4; PBS) for 45 minutes and then washed in PBS containing 0.05% Tween 20.

The conjugates were diluted in 0.5% BSA PBS (anti IgA 1/2000; anti IgG 1/5000 and anti IgM 1/5000) and incubated in the wells for 4 hours at $37^{o}C$ (100ul per well). The colour (OD_{405} minus background) produced after a 2 hour incubation with substrate (100ul of 1mg/ml, Sigma p-nitrophenyl phosphate in 10% diethanolamine hydrochloride, pH 9.6) was measured using a Titertek Multiskan 310C ELISA reader (Flow Laboratories). The sensitivity of this assay enabled

the detection of 1ng/ml of IgG, IgA or IgM. (This had been determined previously by Mrs. D. DiMatteo of the Department of Medicine using radioiodinated immunoglobulins).

II. SPECIFIC METHODS USED FOR THE PREPARATION OF PROTEINS AND ANTISERA

1. Secretory Component

(a) Preparation Of Secretory Component

Secretory component (SC) was purified from human colostrum. Two methods of obtaining SC were used. The first utilized anion exchange and gel chromatography. The second, affinity chromatography.

i) Preparation Of Secretory Component Utilizing Anion Exchange And Gel Chromatography

The first method utilized to prepare SC was based on the methods employed by Newcomb et al (1968), Kobayashi, (1971) and Mestecky et al, (1972).

110mls of pooled colostrum obtained from mothers in the first 4 days post partum was first concentrated to 25mls. It was then defatted. The colostrum was diluted one in two with 0.15M sodium chloride and centrifuged at 20,000 RPM for 60 minutes. The middle layer was retained. The casein was precipitated by lowering the pH to 4.6

with 1M acetic acid and then centrifuging the preparation at 10,000 RPM for 20 minutes. The supernatant was dialysed against 0.01M tri hydroxy methyl amino methane (TRIS) HCl buffer at pH 8.0. Next 50% by volume of saturated ammonium sulphate was added dropwise to the solution which was kept stirring at $4^{o}C$ for 1 hour. This was then centrifuged at 10,000 RPM for 20 minutes. The precipitate was resuspended in 0.01M TRIS HCl pH 8.0 and then dialysed extensively against this buffer.

Ion exchange chromatography on diethyl amino ethyl (DEAE) cellulose (DE52, Whatman) was then performed. The sample was applied to a column of DE52 equilibrated with 0.01M TRIS HCl pH 8.0. Fractions were eluted by stepwise addition of 0.025 M, 0.05M, 0.1M and 2.0M sodium chloride in the 0.01M TRIS HCl pH8.0.

The fractions eluted with 0.05M NaCl contained SC. The fraction eluted with 0.1M NaCl in 0.01M TRIS HCl contained secretory IgA (see section II,2).

The fractions eluted with 0.05M NaCl in 0.01M TRIS HCl pH8.0 were pooled and concentrated using the Amicon diaflo apparatus and a YM10 membrane. Ouchterlony analysis revealed immunoprecipitation against anti SC (Behring OTPL

04/05).

Further purification was performed by Fast Protein Liquid Chromatography (FPLC) on a Mono-Q ion exchange column (Pharmacia). The FPLC purification was performed by P. Ey. After this the preparation was subjected to gel chromatography using a Sephacryl S200 column (Pharmacia). The SC was contained in the main peak eluted off the column. Fractions were analysed by electrophoresis in SDS and the presence of SC was monitored by both immunodiffusion (using a SC specific commercial antiserum: Behring anti SC OTPL 04/05) and ELISA (using an anti SC mouse monoclonal antibody raised in the Department of Medicine, University of Adelaide, from mice immunised with secretory IgA (Section IV,2,a).

Upon electrophoresis in SDS the final preparation yielded a band equivalent to a 75 to 80 kilo dalton polypeptide. It was totally free of immunoglobulin contaminants (determined by ELISA and immunodiffusion). In addition an ELISA was performed to determine if the preparation was contaminated with lactoferrin. This was an inhibition ELISA involving competition by decreasing amounts of lactoferrin (Sigma lactoferrin, L3639) or the SC preparation to

prevent the binding of anti lactoferrin antibodies (Antihuman lactoferrin, Serotec AHP 13) to solid phase bound lactoferrin (section II,1b). The SC preparation contained <.6% by weight of lactoferrin.

This SC was used in the RIAs for serum antigen specific pIgA and total serum pIgA. The concentration of the SC was determined spectrophotometrically using an $E^{1cm}_{1\%}$ at 280nm of 12.7 (Kobayashi, 1971).

ii) Preparation Of Secretory Component Utilizing Affinity Chromatography

Secretory component was also purified from human colostrum according to the method described by Underdown et al (1977) which utilized affinity chromatography on IgM sepharose. The IgM sepharose was prepared as described in section III,2.

50ml of colostrum was centrifuged at 4800g for 1 hour at 4°C. The middle layer was retained. 30ml of this middle layer was added to 270ml 0.01 M citrate 0.02M phosphate pH 6.8 containing 250mg ferrous ammonium sulphate. This was left stirring for 2 hours at 4°C. Then 6ml IgM Sepharose (18mg/ml IgM) was added and 6ml of PBS pH 7.4. This was left stirring gently at 4°C for 16

hours.

The Sepharose was poured into a column and washed with PBS pH 7.4 till the OD of the effluent was that of PBS. After all the unbound protein was eluted the column was washed with 1M KSCN in PBS. The thiocyanate elute was immediately dialysed against PBS at 4°C. After 5 changes of 100 volumes the protein was concentrated and then subjected to fractionation by gel chromatography on a Sephacryl S200 column (Pharmacia).

A single peak of protein was obtained. This was analysed as described in section II,1a(i). Analysis confirmed that the protein was secretory component.

(b). ELISA To Test For Lactoferrin Contamination Of Secretory Component

The wells of microtitre plates were coated with human lactoferrin (Sigma, L3639). 100ul of lactoferrin (5ug/ml) was incubated per well at 4°C overnight. The wells were blocked and washed with PBS Tween 20. Then serial dilutions of 50ul lactoferrin or secretory component from 250ug/ml in PBS mixed with 50 ul of goat anti lactoferrin at a dilution of 1/400 (Serotec AHP 13) were incubated in the wells at 37°C overnight. After washing with PBS Tween 20, 100ul of

a rabbit anti goat IgG alkaline phosphatase conjugate (supplied by P. Ey) diluted 1/1000 was incubated in the wells at 37°C overnight. The colour (OD_{405} minus background) produced after a 5 hour incubation with substrate (100ul of 1mg/ml Sigma p nitrophenyl phosphate in 10% diethonolamine HCL pH 9.6) was measured. This inhibition ELISA revealed that the SC contained < 0.6% by weight of lactoferrin.

(c). Radiolabelling Secretory Component

Three methods of radiolabelling SC were evaluated. The first method was the chloramine T method. Two alternative methods which had been claimed to be less likely to cause damage to proteins during radioiodination were also evaluated.

i) The Chloramine T Method

The chloramine T procedure (McConahey and Dixon, 1966) was followed. 100ug of SC in 0.1ml phosphate buffer pH 7.6 was mixed with 18.5mBq of carrier free ^{125}I (IMS 30, Amersham) and 0.1ml of chloramine T (0.8mg/ml). After 3 minutes at room temperature 0.1mgm of sodium metabisulphite in 0.1ml of phosphate buffer pH 7.6 was added. Both the solution of chloramine T and metabisulphite were freshly prepared. The

labelled SC was then mixed with 2ml of 5% bovine serum albumin (BSA) in 0.1M phosphate buffer, pH 7.6

A 5cm x 1cm column of Sephadex G25 was prewashed with 2ml of 5% BSA in phosphate buffer and then with the phosphate buffer alone. The labelled SC with 2ml of 5% BSA in phosphate buffer was passed through the column. The column was then washed with 5% BSA in phosphate buffer. 1ml fractions were collected. The labelled SC was recovered in 2ml. The specific activity was usually around 5000 cpm per ng. At least 97% of the label was precipitable by 12% tri chloroacetic acid.

ii) The Iodogen Method

This method was based on the procedure described by Fraker and Speck (1978). The iodogen tubes used were prepared by P. Ey. This method eliminates the reduction step at the close of iodination with chloramine T.

Preparation of Iodogen Tubes

1.1mg of Iodogen was dissolved in 25ml of methylene chloride. This stock solution was diluted 1 in 5 in methylene chloride. 50ul of the diluted solution was added to each 1ml eppendorf tube. The tubes were left to dry

overnight in a fume hood. They were stored in the dark at room temperature. These tubes were stable for 6 months (personal communication P. Ey).

Radiolabelling the Secretory Component

30ug of SC in 33ul of 0.1M phosphate buffer pH 7.6 was added to the iodogen coated tube. 5ul (18.5 mBq) of ^{125}I (IMS30, Amersham) was added immediately. The solutions were mixed and the reaction allowed to proceed for 14 minutes at room temperature. Then 80ul of tyrosine was added. The solution was mixed and left to stand for 5 minutes at room temperature. Then 120ul of BSA with phenol red, 0.1mg/ml was added. The latter acted as a marker for free iodine. Next 100ul of cytochrome C (10mg/ml) which is excluded by Sephadex G25 was added. The 338ul sample was then applied to a G25 column previously equilibrated with Tris buffered saline (25mM TRIS, .13M NaCl, 1mM EDTA, .05% azide; TESA). The G25 column had previously been calibrated with radioiodinated IgG. The radiolabelled SC and cytochrome C was eluted in 1.3ml.

This sample was loaded onto a 95cm Sephacryl S200 column (Pharmacia) equilibrated with TESA, 1mM EDTA, 0.1% azide and 25ug/ml BSA. This buffer was pumped through at 0.5ml/minute. 3ml

fractions were collected. The column had previously been calibrated. IgG eluted at fraction 25 and cytochrome C at fraction 37. SC was expected to elute between fraction 28 to 30. 5ul of each fraction were counted. Fractions 27 to 35 contained 75% of the counts. These 9 fractions were pooled. The specific activity was around 5000cpm per ng.

iii) The Bolton And Hunter Method Of Iodinating Secretory Component

This method of radioiodination (Bolton and Hunter, 1973) avoids the direct exposure of the protein to ^{125}I or reagents such as the oxidizing agent chloramine T or the reducing agent metabisulphite. The Bolton and Hunter reagent (Amersham) was used. Prior to use the solvent was removed by evaporation. 120ul of Bolton and Hunter reagent (185mBq per ml) was placed in an eppendorf tube and the solvent evaporated by directing a gentle stream of dry nitrogen onto it for 20 minutes at room temperature. The evaporation was carried out in a well ventilated fume hood. After drying the reagent, 30ug of SC (33.3ul of 0.92mg/ml) was added to the tube. After mixing 277ul of borate buffer (0.2M borate buffer pH 8.5) was added and

the mixture gently agitated for 1½ hours at 4°C. 400ul of 0.2M glycine in 0.1M borate buffer pH 8.5 was then added and left to react for 5 minutes. Then 0.25% gelatin (W/V) was added to make the mixture up to 1ml.

The labelled SC was separated by gel filtration on a Sephadex G25 column previously equilibrated with 0.25% (w/v) gelatin in 0.05M phosphate buffer pH 7.5.

The specific activity of the SC was 1300 cpm per ng. 98% of the label was precipitable by 12% tri chloro acetic acid.

2. Secretory IgA

Preparation Of Secretory IgA

Secretory IgA was prepared from human colostrum as described by Newcomb et al (1968). Secretory IgA was obtained from the same sample of colostrum used in the preparation of SC (section II, 1a(i)). The removal of fat and casein was carried out and ion exchange chromatography on diethyl amino ethyl (DEAE) cellulose (DE52 Whatman) was performed as described in section II,1a(i). The fractions eluted with 0.1M sodium chloride in 0.01M TRIS HCL pH 8.0 contained the secretory IgA (Ouchterlony analysis).

The fractions were pooled, concentrated and then

subjected to gel chromatography on an Sephacryl S300 column (Pharmacia). The flow rate was 0.3ml per minute 4.5ml fractions were collected. Each fraction was analyzed by single radial immunodiffusion (Mancini et al, 1965) using IgA specific antiserum (Behring anti α chain: ORCI 14/15).

As described by Newcomb et al (1968) the second half (the descending limb of the peak) contained secretory IgA. These fractions were pooled concentrated and rerun on the Sephacryl S300 column as described by Newcomb et al. This procedure was repeated. Each fraction of the descending limb of the peak obtained after the third S300 fractionation was analysed separately. The fractions containing pure secretory IgA were pooled and concentrated.

Immunodiffusion, immunoelectrophoresis, ELISA and polyacrylamide gel electrophoresis in SDS confirmed the purity of this preparation.

3. Polymeric IgA

(a) Preparation Of Polymeric IgA

i) Polymeric IgA From Pooled Sera Of Patients With Alcoholic Liver Disease

Polymeric IgA was isolated from the pooled sera of five patients with alcohol induced

cirrhosis adapting the method of Brandtzaeg (1976).

Calcium chloride ($CaCl_2$) was added to 100ml of pooled serum to obtain a 0.09M solution. 0.998gm of $CaCl_2$ was added. When this had dissolved 0.5% weight/volume dextran sulphate (0.5g) was added. The solution was left stirring for 1 hour at room temperature. The lipoprotein precipitate was removed by centrifugation at 10,000 R.P.M. for 15min. To the supernatant saturated ammonium sulphate was added dropwise until the solution was 38% saturated. The solution was left stirring at $4^{o}C$ for 60min. The precipitate of immunoglobulin was obtained by centrifugation at 10,000 R.P.M. for 15min. The precipitate was resuspended in phosphate buffer (0.02M Na_2 HPO_4/KH_2PO_4, pH 8.0) and then dialysed against the buffer. After 5 changes a 55ml solution was obtained.

This was purified by ion exchange chromatography on DEAE cellulose (Whatman, DE52). A 30cm x 5cm column was first equilibrated with 0.02M phosphate buffer pH8.0. The sample was loaded and the column washed with the 0.02M phosphate buffer. Then a stepwise elution with NaCl was performed. Initially, 20mM NaCl then 40mM, 60mM, 80mM and 100mM NaCl in

0.02M phosphate buffer pH8.0 (Na_2HPO_4/KH_2PO_4). The fractions eluted with each change were pooled concentrated and then gel chromatography using Sephacryl S300 (Pharmacia) was performed using a 90 x 1.6cm column. The fractions obtained were analysed by polyacrylamide gel electrophoresis The 80mM eluate contained pIgA. The fractions eluted off the S300 column which contained pIgA were pooled concentrated and then fractionated on a Sephacryl S200 column (Pharmacia). Each fraction eluted was then analysed for immunoglobulin contamination by ELISA and immunodiffusion. Each fraction was analysed by polyacrylamide gel electrophoresis. The fraction containing pIgA were pooled and concentrated. This polyclonal preparation of pIgA was pure as judged by immunodiffusion, ELISA and polyacrylamide gel electrophoresis. This preparation of pIgA was used to determine the sensitivity of the assay for pIgA antibody and was the standard utilized in all the assays for measuring the total serum pIgA in samples of sera.

ii) Polymeric IgA From Sera Of Patients With Myeloma

pIgA was also purified from the sera of 3 patients with IgA myeloma.

Saturated ammonium sulphate was added dropwise to serum until the solution was 40% saturated. The solution was left stirring at 4°C for 1 hour. The precipitated immunoglobulins were obtained by centrifugation at 10,000 R.P.M. for 20 minutes. The precipitate was resuspended in phosphate buffer (0.02M Na_2 HPO_4 KH_2 PO_4 0.2M NaCl 0.01% Na azide, pH 7.6) and dialysed against the buffer. After several changes the sample was concentrated. Gel chromatography was then performed using a 95 x 1.6cm column of Sephacryl S300 (Pharmacia). The buffer was pumped through at 0.3ml per min collecting 4.5ml fractions. Each fraction was analysed by immunodiffusion to determine IgA content and detect contamination with IgG and IgM. The fractions containing IgA were pooled concentrated and then subjected to gel chromatography using a 100 x 1.6cm Sephacryl S200 column (Pharmacia). Each fraction was analysed by immunodiffusion, ELISA and polyacrylamide gel electrophoresis. The fractions containing pIgA which were free of other immunoglobulin contaminants were pooled and concentrated.

(b) Radiolabelling Polymeric pIgA

100ug of pIgA prepared as described in section

3a(i) from the serum of patients with alcohol induced cirrhosis was radiolabelled with ^{125}I using the chloramine T method described by McConahey and Dixon (1966).

Chloramine T (0.8mg.ml) and sodium metabisulphite (2mg/ml) in 0.1M phosphate buffer pH 7.6 were prepared fresh. 100ul of the chloramine T was added to 100ug of pIgA in 290ul phosphate buffer. 18.5mBq (11µl) of ^{125}I (IMS30, Amersham) was added. The solutions were mixed and left to stand for 3 minutes at room temperature. Then 100ul of sodium metabisulphite was mixed in. The labelled pIgA was then mixed with 500ul of phosphate buffered saline pH7.4. The free ^{125}I was removed by dialysis against 6 changes of 1 litre of phosphate buffered saline pH 7.4.

The specific antibody of the labelled pIgA was 6000cpm per ng. This ^{125}I labelled pIgA was used to determine the sensitivity of the SC binding assays.

4. IgG

(a) Preparation Of IgG

This was prepared from pooled normal human sera. The globulin fraction was precipitated by adding ammonium sulphate to a 50% concentration. The sample was centrifuged at 2800 R.P.M. for 20 minutes

at 4°C. The precipitate was resuspended in 0.01M phosphate buffer (Na_2 HPO_4 KH_2 PO_4, pH 8.0) and then dialysed against this buffer at 4°C. After 4 changes the dialysate was centrifuged at 2200 R.P.M. for 10 minutes to remove the fine precipitate which appeared during dialysis.

The supernatant was applied to a diethyl amino ethyl (DEAE)-cellulose (Whatman, DE52) column equilibrated with 0.01M phosphate buffer pH 8.0. The column was washed with this buffer, the fractions of the eluate containing protein were pooled and concentrated using the Amicon diaflo apparatus (YM/10 membrane).

The concentrated sample was then applied to a 95 x 1.6cm upwardly flowing column of Sephacryl S300 (Pharmacia). A single peak of protein was obtained. Ouchterlony, immunoelectrophoresis and ELISA using heavy chain specific commercial alkaline phosphatase conjugates (Kirkegaard Perry Laboratories) showed that this was pure IgG.

5. IgM

(a) Preparation Of IgM From Normal Human Sera

IgM was prepared from human serum. Saturated ammonium sulphate was added dropwise until the

solution was 50% saturated. The solution was left stirring at 4°C overnight. It was centrifuged at 4500 R.P.M. for 30 minutes. The precipitate was resuspended in a phosphate buffer (.02M Na_2 HPO_4, KH_2 PO_4 0.2M NaCl and 0.01% sodium azide pH 7.6) and dialysed against 6 changes of 100 volumes of the buffer over 72 hours.

The IgM was obtained from this by gel chromatography on Sephacryl S300 (a 90 x 1.6cm column equilibrated with the phosphate buffer). Buffer was pumped through at a flow rate of 0.3ml per minute and 4.5ml fractions were collected. Each fraction was analysed by single radial immunodiffusion (Mancini et al, 1965). The first 3 fractions eluted containing IgM were pooled. Six 3ml aliquots of the saturated ammonium sulphate preparations were subjected to gel chromatography in S300.

The first 3 fractions containing IgM from all 6 S300 fractionation were pooled. 60 mls of the pool containing 1.8mg per ml were then further purified by Fast Protein Liquid Chromatography (FPLC) by P. Ey.

20ml of 2.73 mgm/ml was obtained after FPLC. This was applied to a Sephacryl S300 column. Buffer was pumped through at 0.3ml per minute and 7.7ml

fractions collected. A single peak was obtained. This was concentrated and analysed by immunodiffusion, polyacrylamide gel electrophoresis and ELISA. It was free of immunoglobulin contaminants. 55mg IgM was obtained in order to prepare antibodies to IgM.

(b) Preparation Of IgM From Sera Of Patients With Waldenström's Macroglobulinaemia

IgM was also prepared from the sera of 3 patients with Waldenström's macroglobulinaemia.

Gel chromatography using Sephacryl S300 (Pharmacia) was used to purify the IgM from serum. Serum was run onto a 95 x 1.6cm column equilibrated with phosphate buffer (0.02M Na_2 HPO_4, KH_2 PO_4, 0.2M NaCl, 0.01% Na azide, pH 7.6).

Buffer was pumped through at 0.3ml per minute collecting fractions of 4.5ml. Each fraction was analysed by immunodiffusion and ELISA. The first 3 fractions contained pure IgM. This preparation was utilised in the development of the assay for pIgA antibody and to coat the plates in the assay for total pIgA in serum.

6. Preparation Of Anti Light Chain

(a) Preparation Of Light Chain

Light chain was prepared from IgG according to the method described by Williams and Chase (1968).

IgG was purified from pooled normal human as described in section II4. 318mg of purified IgG was obtained from 42mls of pooled normal human serum. The purity was confirmed by immunodiffusion and ELISA. To this 1ml of 2-mercaptoethanol was added so that the final concentration was 0.75M.

Nitrogen was bubbled through this solution for 30 minutes. It was kept cool in ice. Then an equal volume (19ml) of 0.75M iodo acetamide was added. Tri ethyl amine was added dropwise to keep the pH over 8.0. After stirring the solution on ice for 1 hour keeping the pH over 8.0 the reduced IgG was dialysed against normal saline at $4^{o}C$. After 5 changes the solution was concentrated. 12ml containing 24mg/ml protein was obtained. This was dialysed against 1N propionic acid (3 changes of 500ml). 10ml of this OD_{280}1.8 (1/20 dilution) was then fractionated on a Sephadex G75 column (2.5 x 95cm) (Pharmacia) which had previously been equilibrated with 1N propionic acid.

The fractions obtained were dialysed against phosphate buffered saline, PBS pH 7.4 and then analysed by polyacrylamide gel electrophoresis. The fractions containing light chain were pooled and concentrated to 25ml of 1.07mg.ml.

(b) Preparation Of Light Chain Sepharose

20mg of the light chain was coupled to cyanogen bromide activated Sepharose 4B (Pharmacia).

20mg (1mg/ml) of light chain was dialysed against coupling buffer (0.1M $NaHCO_3$, 0.5M NaCl pH 8.3) 2g of CNBr activated sepharose 4B was resuspended in 1mM HCl. The gel was then washed with 500ml of 1mM HCl and then the coupling buffer. The light chain was mixed with the gel in a test tube and placed on a rotary stirrer and rotated end over end for 2 hours at room temperature. Unbound protein was washed away with coupling buffer on a sintered glass funnel. Remaining active sites were quenched with 1M ethanolamine at pH 8.0 for 2 hours. Three washing cycles to remove non covalently adsorbed proteins were used consisting of a wash at pH 4. (0.1M acetate buffer with 1M sodium chloride) followed by a wash at pH 8.0 (0.1M borate buffer containing 1M sodium chloride). The effectiveness of the coupling procedure was checked by determining protein losses in

the various washing steps. The efficiency of the coupling was 90%.

(c) Affinity Purification Of Anti Light Chain Using Light Chain Sepharose

Anti light chain antibodies were prepared by affinity purification of a crude goat anti IgG serum prepared by a previous postgraduate student (Dr. P. McKenzie). (The goats had been immunised with human polyclonal IgG).

Light chain coupled to Sepharose (section II6b) was used for this purpose. The light chain sepharose was poured into a column and equilibrated with 0.1M phosphate buffer ($Na_2 HPO_4/KH_2 PO_4$, pH 7.6).

A 10ml aliquot of the goat antihuman IgG serum was run into the light chain column. The flow through the column was stopped for half an hour. The column was then washed with 0.1M phosphate buffer to elute all the unbound protein. The column was then washed with 3M sodium thiocyanate. The fractions containing protein in the thiocyanate eluate (the anti light chain antibody) were pooled and dialysed against phosphate buffered saline pH 7.4.

Ouchterlony analysis confirmed the presence of anti light chain antibody in the thiocyanate eluate. An ELISA was also performed.

(d) ELISA To Test Anti Light Chain Antibody

Microtitre plates with 96 wells (COSTAR) were used. The wells were coated with either purified IgG, IgM or IgA. 100ul of 5ug per ml of each immunoglobulin in carbonate coating buffer, pH 9.6 was added per well.

After overnight incubation at 4°C the wells were emptied. The wells were blocked with 150ul of 0.5% BSA in PBS for 1 hour. The plates were then washed with PBS Tween 20. The anti light chain in decreasing dilutions from a starting dilution of 100ug per ml in 0.5% BSA PBS was added in duplicate (100ul per well). The anti light chain was incubated in the immunoglobulin coated wells for 3 hours at 4°C. After washing a rabbit anti goat antibody coupled to alkaline phosphatase at 1/400 dilution (supplied by P. Ey) was incubated in the wells (100ul per well) at room temperature overnight. After washing 100ul of phosphate substrate (Sigma p-nitro phenyl phosphate in 10% diethanolamine HCl pH 9.6) was added to each well. The colour OD_{405} obtained after incubation at 37°C for 3 hours was read.

This showed that the anti light chain antibody binding to the three immunoglobulins was identical.

The binding of the immunoglobulins IgG, IgA and IgM to the wells was confirmed by performing an assay in parallel using commercial heavy chain specific anti serum coupled to alkaline phosphatase (Kirkegaard Perry Laboratories).

III. PREPARATION OF ANTI IgM SEPHAROSE COLUMNS USED TO DEPLETE SERUM OF IgM

In view of the strong affinity of IgM for SC it became necessary to remove IgM from serum prior to performing the assay for pIgA antibody (chapter 3). This was done using columns containing anti IgM antibody coupled to Sepharose 4B. This section describes the preparation of the anti IgM antibody, its purification, the preparation of the IgG and IgM sepharose affinity columns used to purify the anti IgM antibody and finally that preparation of the anti IgM sepharose columns.

1. Preparation Of Anti IgM For Sepharose Affinity Columns

(a) The Tryptic Digestion Of Immunoglobulin M To Obtain The Pentameric $(Fc)_{5\mu}$ fragment

The method used to obtain the IgM $(Fc)_{5\mu}$ fragment was based on the method described by Plaut and Tomasi (1970) and Ghose (1971).

The 55mg of IgM obtained (section II5a) was in a buffer containing 25mM TRIS, 0.13 M NaCl 0.1mM EDTA and 0.05% Na azide. The molarity of the solution was increased to 75mM TRIS. To this 150ul of 0.1M $CaCl_2$ was added. The solution was placed in a water bath incubator at 56°C for 5 minutes. 0.5ml of 5mg/ml trypsin was added. After 30 minutes at 56°C a mixture of 3 inhibitors was added. These 3 inhibitors were:

1. Soya bean trypsin inhibitor 2.5mgm
2. Aprotinin 0.75mgm in 1.25ml
3. NPGB in dimethyl formaldehyde (200ul of 0.5mgm per ml)

After the addition of the inhibitors the solution was cooled. It was then applied to a Sephacryl S300 column. Three peaks were obtained off the column. The 3 pools were thought to represent, (1) intact IgM, (2) the $(Fc)_{5\mu}$ fragment, (3) The Fab fragment. The 3

pools were analysed for contamination with light chain by ELISA (section III,1b).

(b) Assessments Of The Purity Of The Presumed IgM $(Fc)_{5\mu}$ Fragment By ELISA

The ability of purified IgM and the 3 pools of protein obtained off the S300 column after the tryptic digest to inhibit the binding of a commercial heavy chain specific anti u conjugate (Kirkegaard and Perry Laboratories) and a goat anti human light chain conjugate (prepared by Ms. J. Remes) to IgM coated on the wells of micro titre plates was assessed.

Costar 96 well microtitre plates were coated with 10ug per ml purified IgM; 100ul per well. The immunoglobulin diluted in 0.05M sodium carbonate buffer, pH 9.6 was incubated in the wells at $4^{o}C$ overnight. The wells were emptied and blocked with 150ul per well of 0.5% BSA in PBS for 1 hour at $4^{o}C$. The wells were then washed with PBS Tween 20.

A starting dilution of the IgM used for the tryptic digest and the 3 pools of protein of 20ug/ml was made in 0.5% BSA PBS. 10 fold dilutions of each in 0.5% BSA PBS were performed. 50ul of each dilution of the 4 protein preparations. (The purified IgM, the presumed intact IgM off the S300 column, the presumed

$(Fc)_{5\mu}$ fragment and the presumed Fab fragment) were added to the IgM coated wells in duplicate. Then 50ul of the alkaline phosphatase conjugates (anti light chain at 1/500 anti u at 1/2500 diluted in 0.5% BSA in PBS were added to each well. This resulted in a starting dilution of 10ug/ml of each of the 4 preparations and 10 fold serial dilutions of each preparation. The end concentration of the conjugates were 1/1000 for anti light chain, and 1/5000 for anti u respectively.

After a 3 hour incubation at $4^{o}C$ the wells were emptied and the plates washed with PBS Tween 20. The colour (OD_{405} minus background) produced after a 2 hour incubation with substrate (100ul of 1mg/ml Sigma p-nitrophenyl phosphate in 10% diethanolamine-HCl, pH 9.6) was measured.

Result

i) Inhibition of binding of anti u to IgM.

The Fab fragment did not inhibit the binding. The original IgM and the $(Fc)_{5\mu}$ fragment pool inhibited the binding of the conjugate to IgM. (50% inhibition 0.7ug/ml and 0.8ug per ml respectively). There was inhibition by the presumed intact or undigested IgM (or possible pooled aggregated $(Fc)_{5\mu}$ complexes) at 2.5ug per ml.

ii) Inhibition of binding of the anti light chain conjugate.

Inhibition occurred with the IgM and the Fab fragment pool (50% inhibition at 0.5ug/ml and 0.4ug/ml respectively). The $(Fc)_{5\mu}$ fragment pool did not produce any inhibition.

The 3 pools were analysed by polyacrylamide gel electrophoresis by P. Ey.

There was no light chain detected in the $(Fc)_{5\mu}$ pool.

This $(Fc)_{5\mu}$ was used to prepare anti serum to IgM in rabbits (section III,1c).

(c) Preparation Of Antiserum To IgM

Rabbits were immunised with the purified $(Fc)_{5\mu}$ fragment. The primary immunisation was with 100ug of $(Fc)_{5\mu}$ fragment in 1ml of PBS pH 7.4 emulsified with complete Freunds adjuvant. Injections were given in 4 subcutaneous sites.

Booster injections of 100ug of the $(Fc)_5$ fragments in 1ml of PBS, pH 7.4 emulsified with incomplete Freunds adjuvant were given 3 weeks later and then at intervals of one to 2 months. The animals were bled 5 weeks after the primary inoculation (2 weeks after the booster) and then a fortnight after each subsequent booster injection. 20ml of blood was obtained from each animal on each occasion. Serum was stored at $-20^{o}C$.

(d) Affinity Purification Of The Rabbit Antiserum Against Human $(Fc)_{5\mu}$

The antiserum raised against $(Fc)_{5\mu}$ fragments was purified by affinity chromatography using IgG sepharose and IgM sepharose adsorbents. The

preparation of the IgM and IgG sepharose is described in section III,2a and III,2c respectively.

Anti light chain cross reactivity in the rabbit antiserum was removed by running the serum into an IgG Sepharose column previously equilibrated with 0.1M phosphate buffer pH 7.6. After allowing the serum to interact with the IgG sepharose for 45 minutes the column was washed with 0.1M phosphate buffer pH 7.6 and the protein eluted collected and concentrated. This protein had anti u activity on analysis by Ouchterlony and immunoelectrophoresis.

This protein was then run into the IgM Sepharose adsorbent column. After allowing an interaction of 45 minutes the column was washed extensively with 0.1M phosphate buffer pH 7.6 to remove unbound protein. The anti IgM specific antibody was then eluted by washing the column with 3.0M sodium thiocyanate. The protein obtained was dialysed against PBS pH 7.4 and analysed by Ouchterlony and immunoelectrophoresis. It was IgM specific. This u chain specific antibody was then coupled to Sepharose 4B (section III,3).

2. Preparation Of Sepharose Columns Used To Affinity Purify The Rabbit Antiserum Against Human (Fc) 5μ

(a) The Coupling Of Human IgM To Cyanogen Bromide Activated Sepharose 4B

IgM purified by precipitation with ammonium sulphate and chromatography as described (section 5) was coupled to commercial cyanogen bromide activated Sepharose 4B (Pharmacia).

3g of Sepharose 4B was resuspended in 100ml 1mM HCl. The gel was then washed with 600ml of 1mM HCl. Next it was mixed with coupling buffer (0.1M $NaHCO_3$ 0.5M NaCl, pH 8.3).

32mgm of IgM was dialysed against coupling buffer and then concentrated to 5ml. The protein was mixed with the gel in a test tube and rotated end over end for 2 hours at room temperature. The ratio of gel to protein during the process was 1g of swollen gel to approximately 3mls of protein solution. Unbound protein was washed away with 50ml coupling buffer. Remaining active sites on the gel were quenched with 1M ethanolamine at pH 8.0 for 2 hours. The non covalently adsorbed protein was removed by washing the gel. The first wash was with 50ml of 0.1M acetate buffer with 1M sodium chloride pH 4.0 followed by a

wash with 50ml 0.1M sodium bicarbonate and 1M sodium chloride pH 8.0. A third wash with the acetate buffer and a fourth with the sodium bicarbonate buffer was performed. The protein loss in each washing step was estimated. Approximately 2mg protein was lost leaving 30mg coupled to 10ml sepharose.

The sepharose was then poured into a column and then washed with 0.1M phosphate buffer (0.1M Na_2 HPO_4, KH_2PO_4 pH 7.6).

(b) Testing The IgM Sepharose Column

5ml of goat anti human IgM serum was run into the IgM sepharose column and washed through with 0.1M phosphate buffer. The protein bound to the column was eluted with 3M sodium thiocyanate in 0.1M phosphate buffer. The eluate was immediately dialysed against repeated changes of PBS at $4^{o}C$. 1.15mg/ml protein was eluted. This protein was analysed by Ouchterlony analysis. Precipitation lines against IgM developed.

This IgM sepharose was used in the purification of the rabbit antibody to IgM.

(c) The Coupling Of IgG To Cyanogen Bromide Activated Sepharose

Polyclonal IgG was prepared from pooled human

sera (section II,4). The IgG was precipitated by ammonium sulphate (50% saturated) and purified by chromatography on DEAE cellulose (Whatman, DE52) followed by gel chromatography on Sephacryl S300 (Pharmacia) as described in section II,4. The purity of the preparation was confirmed by immunodiffusion and ELISA.

30mg of the IgG was coupled to 3g cyanogen bromide activated Sepharose 4B (Pharmacia). The method was identical to that outlined for the coupling of IgM to sepharose.

(d) Testing The IgG Sepharose Affinity Column

The IgG Sepharose adsorbent was poured into a column and washed extensively with 0.1M phosphate buffer (Na_2 HPO_4 KH_2 PO_4, pH 7.6). 9ml of a goat anti human IgG (prepared by P. McKenzie) was run into the column and the column was washed with phosphate buffer until all the unbound protein had eluted. The bound protein was eluted with 3.0M sodium thiocyanate and dialysed extensively against PBS pH 7.4. Ouchterlony analysis confirmed that the protein eluted with thiocyanate was antibody to IgG.

The IgG Sepharose was utilized in the affinity purification of the rabbit anti IgM (section III,1d).

3. Coupling Of Rabbit Anti Human IgM To Cyanogen Bromide Activated Sepharose 4B

Two preparations of affinity purified rabbit anti human IgM comprising 12ml of 1.4mg/ml protein and 14ml of 1.3mg/ml protein were combined, dialysed against coupling buffer and then coupled to 2gm of cyanogen bromide activated Sepharose 4B (Pharmacia). The coupling procedure was as outlined in the preparation of the IgM Sepharose.

This anti IgM Sepharose was poured into 2ml disposable polypropylene columns (Amicon C/N AF25). There was 1mg of antibody per ml of gel.

4. Assessing The Effectiveness Of Anti IgM Sepharose Affinity Columns In Depleting Serum Of IgM

The effectiveness of the 2ml anti IgM Sepharose columns in removing IgM from serum was determined by testing the ability of the columns to totally deplete aliquots of 2ml, 1ml or 0.5ml of serum, of IgM.

Aliquots of serum were run into the columns which had previously been equilibrated with 0.1M phosphate buffer (Na_2 HPO_4 KH_2 PO_4 pH 7.6). After a 45 minute incubation at room temperature the columns were washed with phosphate buffer collecting 0.5ml aliquots.

Unadsorbed protein was all collected in the first 5ml (for the 0.5ml and 1ml aliquots) and 6ml (for the 2ml aliquots of serum). These unadsorbed proteins were analysed by immunodiffusion and ELISA. The efficacy of the columns in removing IgM from even 2ml aliquots of serum was shown by the absence of IgM (<1ng/ml) in the effluent sera. The IgG and IgA content was unchanged.

The bound protein was eluted with 3M sodium thiocyanate and immediately dialysed against phosphate buffer. After 5 changes the protein was analysed by ELISA. This revealed that this fraction contained IgM but no IgA or IgG.

Although the 2ml anti IgM Sepharose columns were shown to have the capacity to completely deplete up to 2ml of serum of IgM, in all the assays described in subsequent chapters the 2ml anti IgM columns were used to deplete 0.5ml aliquots of serum of IgM.

IV. ALKALINE PHOSPHATASE LABELLED CONJUGATES USED TO DETECT POLYMERIC IgA ANTIBODY

An attempt was made to substitute the radioimmunoassay for pIgA antibody with an enzyme linked immunosorbent assay (Chapter 4, section III). Initially the use of alkaline phosphatase labelled SC

was evaluated. As this approach failed the use of an alkaline phosphatase labelled anti SC antibody to detect the binding of SC to pIgA was evaluated. This section describes the preparation of the enzyme linked SC and anti SC.

1. Alkaline Phosphatase Labelled Secretory Component

Secretory component prepared from human colostrum (Chapter 2, section II,1,a,(i)) was labelled with alkaline phosphatase (Bovine intestinal mucosa Type VII-S; Sigma Chemical Co., St. Louis, Ma, USA: Product No. P-554, Lot No. 122F-8030).

200ug of the enzyme in 3.2M$(NH_4)_2$ SO_4 and 1mM $MgCl_2$, 1mM $ZnCl_2$ (5.5mg/ml) was mixed with 200ug of SC (in 300ul of PBS) and then dialysed overnight against 2 changes of 2l of PBS containing 0.5mM $MgCl_2$ and 1.25uM $ZnCl_2$. Then 100ul of 0.25% glutaraldehyde was added dropwise to the sample and the solutions were mixed for 3 hours at room temperature in the dark. Then 200ul of 2M glycine pH7 was added. The sample was then dialysed against 2l of PBS pH7.4 containing 0.5mM $MgCl_2$ and 1.25uM $ZnCL_2$. After this 0.1 volume of 20mg/ml BSA was added. The conjugate was diluted in 0.05M Tris and 1mM $MgCl_2$ (final concentration 100ug/ml) and stored in aliquots at -20^{oC}.

The experiments performed with the alkaline phosphatase labelled SC are described in Chapter 4, section III,1.

2. The Alkaline Phosphatase Labelled Mouse Monoclonal Anti Secretory Component Antibody

The use of an alkaline phosphatase labelled anti SC antibody to detect the binding of SC to solid phase pIgA was evaluated as a means of detecting antigen specific pIgA antibody (Chapter 4, section III,2).

The preparation of the alkaline phosphatase labelled anti SC is described in this section. These experiments were performed in collaboration with Dr. P. Ey.

(a) Source Of The Monoclonal Anti Secretory Component

An anti SC mouse monoclonal antibody was raised in mice immunised with secretory IgA. (This antibody was prepared by Hohmann et al in the Department of Medicine and is now marketed by Silenus; Catalogue No. 12HS PP01 AMD-HSC).

The anti SC antibody was obtained from mouse ascites fluid using anion exchange chromatography on DEAE cellulose (Whatman, DE52) followed by gel chromatography in Sephacryl S300 (Pharmacia) to obtain

the globulin fraction.

(b) Testing The Globulin Fraction Of Mouse Ascites Fluid For Anti Secretory Component Activity

The globulin fraction was tested for SC binding capacity by coating trays with SC (100ul/well of 5ug/ml in carbonate coating buffer, pH 9.6). After overnight incubation, blocking and washing, the anti SC was incubated in a series of dilution in BSA PBS in the wells (overnight at room temperature). A rabbit anti mouse alkaline phosphatase conjugate (supplied by P. Ey) was used to detect the binding of the mouse anti SC to SC bound to the wells. SC could be detected using a 1/100,000 dilution of the anti SC globulin fraction.

(c) The Preparation And Evaluation Of the Anti SC Alkaline Phosphatase Conjugate

The mouse anti SC was then conjugated to alkaline phosphatase by P. Ey. In a series of experiments the optimum condition for using the conjugate were found.

The optimum dilution was 1/500 in BSA PBS with an overnight incubation of the anti SC at room temperature. The optimum incubation conditions for the substrate (Sigma p nitro phenyl phosphate in 10% diethanolamine buffer) was 6 to 8 hours at $37^{o}C$.

This alkaline phosphatase labelled anti SC antibody was used in the experiments described in Chapter 4, section III,2.

V. MATERIAL AND METHODS USED IN THE STUDIES PERFORMED IN NORMAL VOLUNTEERS AND PATIENTS

1. Collection And Storage Of Samples Of Intestinal Fluid And Serum

Intestinal fluid was obtained from the upper jejunum using either a disposable SALEM gastrointestinal double lumen sump tube (Brunswick Co., USA) or an "ANPRO AN20 Andersen" Tungsten weighted sump tube (HW Andersen Products, Oyster Bay, New York, USA).

Fluoroscopy was used to position the tube. Only samples with a pH>7 were stored. Samples were collected on ice, centrifuged and stored in aliquots at -70^{o}C until the assays were performed.

Serum samples were stored in aliquots at -20^{o}C.

2. Typhoid Vaccine

(a) Oral Typhoid Vaccination

Three forms of the oral typhoid vaccine salmonella typhi Ty21a were used in the studies

described in subsequent chapters.

i) Gelatin Capsules Containing The Vaccine

Seven volunteers received gelatin capsules supplied by Professor R. Germanier of the Swiss Serum Institute Berne (chapter 6, section 1). The capsules had been stored at $4^{o}C$ and on previous studies carried out by Professor Germanier contained a minimum of 10^{9} live organisms (personal communication).

ii) Liquid Formulation Of The Vaccine

A further 21 volunteers (chapter 6, section 1) and the patients with alcoholic liver disease who were given the oral typhoid vaccine (chapter 7) received varying doses of the vaccine in liquid form prepared in Adelaide by Professor Rowley et al (Department of Microbiology, University of Adelaide). Ty21a was grown in Brain Heart Infusion Broth (BHIB) for 18 hours and galactose (final concentration 0.001%) was added 4 hours before harvest. The bacteria were harvested by centrifugation and resuspended in the appropriate concentrations in saline. Viable counts of bacteria were determined by plating on nutrient agar.

iii) Lyophilized Vaccine

Some volunteers were given a Ty21a vaccine prepared by Dr. G. Boehm of Enterovax Research Pty. Ltd., Adelaide, and supplied in the form of lyophilized oral doses each comprising 10^{11} viable organisms. The vaccine was resuspended in 50ml of 0.9% sodium chloride before it was given.

(b) Parenteral Typhoid Vaccine

The parenteral vaccine used in the study described in chapter 6, section 2 was the one commercially available (Commonwealth Serum Laboratories) in Australia. Each dose consisted of $5x10^8$ smooth Salmonella typhi organisms that had been heat killed and was administered in 0.5ml as a subcutaneous injection.

(c) Hybrid Cholera-Typhoid Vaccines

Some of the volunteers were given live, oral hybrid cholera-typhoid vaccines (Chapter 6, section II). These vaccines were developed by Professor Derrick Rowley's group. Molecular biologists in the Department of Microbiology developed several clones inserting DNA coding for expression of vibrio cholerae lipopolysaccharide into the typhoid vaccine. All the cloned vaccines (V487, Ex210 and Ex363) given to the volunteers in the studies described in Chapter 6

(Section II) expressed typhoid lipopolysaccharide.

3. Enzyme Linked Immunosorbent Assays For Antigen Specific Antibody In Serum And Intestinal Fluid

Antibody (other than serum pIgA antibody) to typhoid lipopolysaccharide (LPS), Escherichia coli lipopolysaccharide (E.coli LPS), gliadin and Campylobacter antigens was measured in the serum and intestinal fluid of normal volunteers and patients by enzyme linked immunosorbent assay (ELISA).

Polyvinyl microtitre plates containing 96 wells (Costar Data Packaging Corporation, Cambridge, Massachusetts) were used for all the assays.

(a) Antigens

i) Typhoid Lipopolysaccharide

In initial studies (chapter 6, section II) typhoid LPS was obtained from heat killed Salmonella typhi using the phenol water extraction method of Westphal et al (1952); it was double extracted to reduce impurities.

Subsequently Salmonella typhi Ty2 LPS (Sigma, Catalogue No 2595) that had been linked to methylated bovine serum albumin was used.

ii) Escherichia coli lipopolysaccharide

This was a commercial E.coli LPS (Difco Laboratories 055 B5).

This was used to study antibody to E.coli LPS in patients with alcoholic liver disease (chapter 7), Crohn's disease (chapter 8) and normal controls (chapters 7 and 8).

iii) Gliadin

Gliadin, the alcohol soluble extract of gluten (Sigma Chemicals) was used in the study of patients with coeliac disease (Chapter 8, section 1).

iv) Campylobacter Antigens

Campylobacter jejuni strains PEN1, PEN2 and PEN3 were used as these were the strains used in the study of Blaser and Duncan (1984). The 3 strains were kindly supplied by Mr. Walter Woods of the School of Pharmacy of the South Australian Institute of Technology. The antigens were prepared by Ms. J. Lanser of the Institute of Medical and Veterinary Science, Adelaide as described by McCoy et al (1975). Equal amounts of each of the 3 antigen preparations were diluted in 0.5M sodium carbonate buffer pH9.6 and combined to yield a total protein content of

5ug/ml.

(b) Description Of The ELISA For Antigen Specific Antibody In Serum And Intestinal Fluid

Micro titre plates were coated with the respective antigens by incubating 100ul of the antigen in each well at 4°C overnight in 0.1M sodium carbonate/bicarbonate coating buffer pH9.6 [10ug/ml typhoid LPS; 5ug/ml S.typhi Ty2 LPS (Sigma) that had been linked to methylated bovine serum albumin; 10ug/ml E.coli LPS; 5ug/ml Campylobacter antigens]. The exception to this was gliadin, the alcohol soluble extract of gluten which was attached to the plates by dissolving it in 70% ethanol at a concentration of 5ug/ml, adding 100ul per well and evaporating the solvent to dryness overnight at room temperature.

After incubating the plates with the antigen at 4°C overnight the wells were emptied and 150ul of 0.5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) left in them at room temperature for 45 minutes. The plates were then washed with PBS containing 0.05% Tween 20.

100ul serial doubling dilutions of the serum or intestinal fluid were added to each well. These samples were incubated overnight at 4°C. After washing

and drying the binding of antibody in the samples was determined using antisera coupled to alkaline phosphatase which produced a colour change when a phosphate substrate was added.

A goat anti human light chain conjugate was used to detect antibody in all immunoglobulin classes (prepared by J. Remes in the Department of Medicine). Commercial goat anti human IgA, IgG or IgM heavy chain specific antisera coupled to alkaline phosphatase (Kirkegaard and Perry Laboratories) were used to detect antibody in the IgA, IgG and IgM class repectively. The conjugates were diluted in 0.5% BSA in PBS (anti light chain 1/2000, anti IgA 1/2000, anti IgG 1/5000, anti IgM 1/5000) 100ul of the conjugate was incubated in each well at 37^{o}C for 4 hours. After washing, 100ul of the substrate (1mg/ml, Sigma p-nitrophenyl phosphate in 10% diethanolamine HCl buffer, pH9.6) was added to each well. The substrate was incubated in each well for 3 hours at 37^{o}C. The colour produced (OD_{405} minus background colour produced by the binding of the conjugate directly to the antigen coated wells) was read using a Titertek Multiskan 310C. ELISA reader (Flow Laboratories).

As 100ul volume dilutions of samples were added to each well, the antibody results are expressed as

antibody units per 100ul. One unit of antibody was defined as that producing an OD_{405} of 0.15 after background subtraction. The immunoglogulin content on intestinal fluid is variable therefore the antibody results in intestinal fluid are expressed in terms of units of antigen specific antibody per mg of immunoglobulin in the samples as determined by single radial immunodiffusion assays (LaBrooy et al, 1980).

4. Immunodiffusion Assays

Total immunoglobulin levels in serum and intestinal fluid were measured by single radial immunodiffusion (Mancini et al, 1965) using heavy chain specific antisera (Behring anti IgA α chain; ORCI 14/15; anti IgG γ chain ORCM 14/15; anti IgM μ chain ORCK 14/15). The serum standard was a commercial preparation (Behring standard human serum ORDT 06/07). For intestinal IgA estimation the standard was secretory IgA purified from human colostrum.

5. Statistical Analysis

The Mann Whitney U Test (two tailed) was used to analyse the differences between the patients and normal controls in respect of antigen specific antibody and the concentration of pIgA and total IgA, IgG and IgM.

Statistical analysis of the differences between levels of antibody to typhoid before and after vaccination were performed using Student's paired t test.

The test used to determine the relationship between serum pIgA antibody, total serum IgA antibody and intestinal antibody was the Spearman Rank correlation coefficient.

VI. MISCELLANEOUS REAGENTS USED IN EXPERIMENTAL WORK

1. Antisera And Alkaline Phosphatase Conjugates Supplied By P. Ey

Several anti sera and anti sera coupled to alkaline phosphatase used in experiments described in Chapter 2 to 4 were supplied by Dr. P. Ey.

They were:

a. A rabbit anti goat antibody directed against goat IgG, IgM and IgA.
b. A rabbit anti goat IgG alkaline phosphatase conjugate.
c. A goat anti human IgM anti serum.
d. A goat anti rabbit anti serum.
e. Purified mouse IgM.
f. Purified mouse IgG.
g. Mouse serum.

2. Alkaline Phosphatase Conjugate Supplied By Ms. J. Remes

An anti light chain antibody coupled to alkaline phosphatase was supplied by Ms. J. Remes of the Department of Medicine.

CHAPTER 3

DEVELOPMENT OF THE ASSAY

1. INTRODUCTION

This chapter describes the development of the assay for antigen-specific polymeric IgA (pIgA) antibody in serum utilising the affinity of secretory component (SC) for pIgA.

In this introduction the methods used to perform the experiments described in this chapter are described. The description of the assay for antigen specific pIgA antibody in its final form is then presented. Experiments performed to assess the binding of SC to immunoglobulins and confirm the validity of the assay are presented. Strategies evaluated in attempting to prevent IgM antibody interfering with the measurement of pIgA antibody are described including the successful use of anti IgM antibody coupled to Sepharose to deplete aliquots of serum of IgM. Experiments performed to optimise the assay and determine the sensitivity of the assay are presented .

In all the experiments described polyvinyl micro titre plates with 96 wells (COSTAR, Data Packaging Corp., Cambridge, Mass, USA) were used. The coating buffer was a sodium carbonate buffer (0.05M sodium

carbonate, pH 9.6). The wells were usually coated by incubating a 100ul solution of the immunoglobulin in coating buffer in each well at 4°C overnight. The wells were then emptied and 150ul of RIA grade 0.5% bovine serum albumin (BSA) in phosphate buffered saline (.04M phosphate, 0.15M sodium chloride pH 7.4; PBS) left in them for 45 minutes at room temperature to block the unbound sites. In all experiments the washing solution used was a solution of PBS containing 0.05% Tween 20, pH 7.4. The binding of the immunoglobulin to plates was confirmed by performing an ELISA in parallel with the RIA using heavy chain specific commercial anti sera coupled to alkaline phosphate (Kirkegaard Perry Laboratories). This assay has been described (Chapter 2, section I,6). The alkaline phosphatase conjugates were diluted in 0.5% BSA in PBS (anti α 1/2000; anti γ 1/5000; anti u 1/5000). 100ul of the conjugate was incubated in each well at 37°C for 4 hours. After washing, 100ul of the substrate (1mg/ml, Sigma p-nitrophenyl phosphate in 10% diethnanolamine-HCL buffer, pH 9.6) was added to each well. The substrate was incubated in each well for 3 hours at 37°C. The OD_{405} minus background produced was read using a Titertek Multiskan 310 C ELISA reader (Flow Laboratories).

In all the experiments described ^{125}I labelled SC was diluted in RIA grade 0.5% BSA PBS. 100ul of the labelled SC was incubated in each well at 4°C. In the initial experiments the ^{125}I labelled SC was incubated in the wells for 4 hours at 4°C. In later experiments the incubation was allowed to proceed for 24 hours. The plates were washed 6 times (see section V,1) after the SC incubation. The radioactivity bound to individual wells cut from the plate was measured using an LKB 1282 Compugamma gamma counter.

II. DESCRIPTION OF THE ASSAY FOR ANTIGEN SPECIFIC POLYMERIC IgA ANTIBODY IN SERUM

(1) Removal Of IgM From Serum Samples

500ul aliquots of serum were totally depleted of IgM using 2ml affinity columns of anti IgM antibody coupled to Sepharose 4B. Each 2ml anti IgM column was equilibrated with 0.1M phosphate buffer, pH 7.6 ($Na_2 HPO_4$ $KH_2 PO_4$) prior to the application of serum. After a 40 minute incubation at room temperature, unadsorbed proteins were eluted by washing the column with phosphate buffer and collected in essentially 100% yield in the first 5ml (giving a 10 fold dilution of the original sample).

(2) Assay For Antigen Specific pIgA Antibody

The wells of micro titre plates (COSTAR) were

coated with antigen diluted in 0.05M sodium carbonate buffer, pH 9.6 (100ul per well). After overnight incubation at 4°C the unbound sites were blocked with 0.5% BSA in PBS for 45 minutes at room temperature (150ul per well) and then washed 10 times with PBS containing 0.05% Tween 20.

Duplicate two-fold serial dilutions of the IgM depleted sera (100ul) in BSA-PBS diluent were incubated in each well overnight at 4°C. The plates were washed with PBS-Tween and then incubated with ^{125}I labelled SC (10ng/well) in 100ul of BSA-PBS diluent at 4°C overnight. The plates were washed 6 times and the radioactivity bound to individual wells cut from the plate was measured using an LKB 1282 Compugamma gamma counter. The end point of the assay was defined as the dilution (=1 Unit) giving 4 times background binding (the latter being the binding of SC to antigen in the absence of serum or antibody). Results are expressed as units of pIgA antibody per 100ul.

The complete removal of IgM from the aliquot of serum was confirmed by performing an ELISA in parallel using heavy chain specific anti sera coupled to alkaline phosphatase (Kirkegaard Perry Laboratories) and single radial immunodiffusion (Mancini et al,

1965).

III. STUDIES OF SECRETORY COMPONENT BINDING

1. BINDING OF SECRETORY COMPONENT TO POLYMERIC IgA AND OTHER IMMUNOGLOBULINS

The clarification of the pattern of binding of labelled SC to immunoglobulins was of critical importance to the development of the assay. The experiments described here were performed to determine if radiolabelled SC would bind to purified immunoglobulins bound to the wells of microtitre plates and to immunoglobulins in the liquid phase. These experiments revealed that labelled SC would bind to pIgA and IgM. To demonstrate that the SC was binding to pIgA and not to contaminants, antibodies specific for IgA and IgM were each tested for their capacity to block the SC binding.

(a) The Binding Of Radiolabelled SC To Immunoglobulins Bound to Micro Titre Plates

The binding of 10ng ^{125}I labelled SC to pIgA, IgM, secretory IgA, monomeric IgA and IgG adherent to the wells of microtitre plates was examined. To confirm that the immunoglobulins were bound to the plate an ELISA was performed using heavy chain specific antisera (Chapter 3, section I).

IgM was purified from the serum of patients with Waldenström's macroglobulinaemia, pIgA from sera of patients with IgA myeloma and sera of patients with alcoholic liver disease. Secretory IgA was purified from human colostrum. IgG and monomeric IgA were prepared from normal sera (see Chapter 2). The SC binding assay was performed as described in the introduction to this chapter.

Radiolabelled SC bound to myeloma pIgA and IgM but not to IgG (Figure 3.1).

Radiolabelled SC bound to non myeloma pIgA and IgM but not to secretory IgA or IgG (Figure 3.2). Labelled SC did not bind to monomeric IgA.

These experiments demonstrated that SC would only bind to pIgA and IgM but not to secretory IgA, monomeric IgA or IgG when these immunoglobulins were used to coat the plates. The binding of SC to IgM appeared to be much stronger than to pIgA (Figures 3.1 and 3.2).

(b) <u>The binding Of Radiolabelled SC To Immunoglobulins Coupled To Plates Coated With Anti Light Chain Antibody</u>

The intended purpose of the SC binding assay was the measurement of pIgA antibody bound to

antigen coated wells, not the measurement of pIgA bound directly to wells. In an attempt to determine if the binding of pIgA to SC could be adversely affected by prior binding of the pIgA to an antigen coated well the binding of SC to immunoglobulins attached to plates through their binding to anti light chain coated on the wells was examined. The binding of labelled SC to pIgA, IgM, IgG and secretory IgA attached to plates through binding to anti light chain antibody was examined.

Micro titre plates were coated with anti light chain (Chapter 2, section II,6; for preparation) by incubating 100ul of 10ug/ml of anti light chain antibody in sodium carbonate buffer in each well overnight at $4^{o}C$. After washing the plates were blocked with 0.5% BSA-PBS (150ul/well) for 45 minutes and then washed. Serial doubling dilutions of the immunoglobulins (pIgA, IgM, secretory IgA and IgG) from 100ug/ml in BSA PBS were incubated in the well overnight at $4^{o}C$. The binding of the immunoglobulins to the anti light chain coated wells was confirmed by an ELISA performed in parallel.

Labelled SC was incubated in the wells for 4 hours at $4^{o}C$ and the counts bound to the well measured.

Radiolabelled SC bound to pIgA and IgM but not to monomeric IgA or IgG bound to the anti light chain coated wells (Figure 3.3). This result also suggested that labelled SC could not only bind to pIgA bound directly to wells but could also bind to pIgA antibody bound to antigen coated wells.

(c) The Inhibition of Binding Of Secretory Component To Polymeric IgA Or IgM By Immunoglobulins In The Liquid Phase

Having demonstrated that SC would bind to solid phase pIgA the capacity of SC to bind to soluble pIgA was tested in order to further validate the use of SC binding assays for pIgA.

Experiments to investigate the capacity of purified immunoglobulins in the liquid phase to inhibit the binding of ^{125}I labelled SC to plates coated with pIgA or IgM were performed.

Microtitre plates were coated with pIgA or IgM. After overnight incubation, blocking and washing, serial dilutions of immunoglobulin in 50ul of 0.5% BSA-PBS were added to each well. Then 50ul of BSA-PBS containing ^{125}I labelled SC was added and mixed with the immunoglobulin in the liquid phase. After incubation at $4^{o}C$ for 4 hours the wells were emptied, the plates washed and the radioactivity bound to the plates was

measured.

The binding of SC to wells coated with pIgA was inhibited by pIgA or IgM in the liquid phase (Figure 3.4). The binding of SC to wells coated with IgM was also inhibited by pIgA or IgM (Figure 3.5).

Secretory IgA, IgG or monomeric IgA in the liquid phase did not inhibit the binding of SC to plates coated with pIgA or IgM. In the liquid phase pIgA was more effective than IgM in inhibiting the binding of SC in contrast to their binding of SC when attached to plates (Figure 3.4 and 3.5). It is possible that this was due to covalent stabilisation of the pIgA-SC complexes by disulphide exchange (Brandtzaeg, 1977).

(d) <u>The Specificity Of Binding Of Secretory Component: Inhibition Of Secretory Component Binding With Specific Anti Sera</u>

The specificity of the binding of SC to pIgA and IgM was confirmed using an ELISA in which the binding of SC to these immunoglobulins was inhibited with heavy chain specific anti sera. These experiments were performed using the enzyme linked mouse monoclonal anti SC which had been coupled to alkaline phosphatase by P. Ey (Chapter 4).

Micro titre plates were coated with pIgA or IgM 5ug/ml, 100ul per well. After overnight incubation at 4°C the wells were blocked and washed with PBS Tween. 100ul serial dilutions of purified rabbit anti IgM (Chapter 2, section III,1) or sheep anti IgA (Silenus) diluted in 0.5% BSA-PBS were incubated in each well coated with IgM or pIgA respectively, for 2½ hours at room temperature. Some pIgA and IgM coated wells did not have anti serum added (positive control). After washing, 100ul of 5ug/ml SC in BSA-PBS was left in each well for 4 hours at room temperature. The plates were washed and 100ul of the anti SC conjugate diluted 1/500 in BSA-PBS left in each well overnight at room temperature. After washing 100ul substrate (1mg/ml Sigma p nitrophenyl phosphate in 10% diethanolamine-HCl, pH 9.6) was left in each well at 37°C. The OD_{405} at 6 hours was read.

The controls were wells coated with pIgA and IgM to which no SC was added. There was no direct binding of the anti SC conjugate to pIgA or IgM coated wells.

The binding of the anti sera to the immunoglobulins was confirmed using a goat anti rabbit anti serum alkaline phosphatase conjugate (Chapter 2, section VI) to detect the binding of

rabbit anti IgM to IgM and a rabbit anti sheep conjugate (Chapter 2, section VI) to detect the binding of sheep anti IgA to pIgA. Another control detected the background binding of anti SC to the anti IgA or anti IgM anti sera. The OD_{405} obtained was subtracted from the OD read when SC had been added to give the results obtained.

Exposure of IgM coated wells to saturating amounts of anti-IgM or of pIgA coated wells to anti IgA antibodies totally inhibited the binding of SC.

In contrast exposure of IgM coated wells to saturating amounts of anti IgA and of pIgA coated wells to anti IgM antibodies, did not inhibit the binding of SC (Figures 3.6 and 3.7).

These experiments confirmed that the binding of SC was occurring specifically to pIgA.

(e) Variability Of Binding Of Secretory Component To Different Preparations Of pIgA And IgM

Only small differences were observed in the binding of SC to different preparations of pIgA (range: 8 to 12% of total counts added) and IgM (range: 24 to 30% of counts added). These included a polyclonal IgM from normal serum, 3 monoclonal IgM proteins derived from sera of

patients with Waldenström's macroglogulinaemia, 3 monoclonal pIgA preparations from myeloma sera and 2 polyclonal preparations from sera of patients with alcohol induced liver disease. Only one preparation a monoclonal myeloma pIgA, failed to bind SC for unknown reasons.

2. Binding Of Secretory Component To Antigen Specific Polymeric IgA

Having demonstrated that ^{125}I labelled SC would bind pIgA, experiments were performed to detect antigen specific pIgA antibody in the serum of human volunteers. The SC binding assay was successfully used to detect pIgA antibody to typhoid lipopolysaccharide in volunteers given the oral typhoid vaccine Ty21a (Chapter 6) and to detect pIgA antibody to gliadin in the serum of patients with coeliac disease (Chapter 8). Two methods were used to confirm that the SC binding was detecting pIgA to these antigens.

(a) Fractionation Of IgM Depleted Samples Of Serum By Gel Chromatography In Order To Confirm That Secretory Component Bound To Polymeric IgA Antibody

To confirm that ^{125}I labelled SC bound specifically to pIgA antibody a serum sample

totally depleted of IgM from a patient with coeliac disease which contained 1200 units of pIgA antibody to gliadin was fractionated on a Sephacryl S200 column. Each fraction was tested for antibody to gliadin using ^{125}I labelled SC to detect the pIgA antibody and an ELISA to detect the total IgA antibody (monomeric and pIgA) to gliadin in the same fraction.

SC binding was associated with fractions containing pIgA antibody and not in fractions containing monomeric IgA (Figure 3.8).

(b) Ablation Of Secretory Component Binding Capacity By Anti IgA Antibody Sepharose Affinity Columns

Further confirmation that the ^{125}I labelled SC was binding to pIgA antibody in the IgM depleted serum samples was obtained by passing these samples through an anti IgA sepharose affinity column.

A sample from a patient with coeliac disease (which had been depleted of IgM) which had 1200 units of pIgA antibody per 100ul to gliadin in the SC binding assay for pIgA antibody to gliadin was sent through an anti IgA sepharose affinity column. The capacity to bind SC was removed.

A sample from a healthy volunteer who had a pIgA serum antibody response to vaccination with

the oral typhoid vaccine Ty21a (1000 units per 100ul of pIgA antibody to typhoid LPS) was also sent through an anti IgA column. The SC binding assay for antibody was repeated on the protein which did not bind to the column. No SC binding occurred.

An ELISA for IgG antibody to gliadin and typhoid LPS in the respective samples was also performed. The level of IgG antibody to gliadin and typhoid LPS respectively was not changed by passage of the sample through the anti IgA column.

IV. PREVENTING IgM INTERFERING WITH THE DETECTION OF POLYMERIC IgA

The experiments described in the previous section revealed that SC had affinity for solid phase IgM as well as pIgA and that the affinity of SC for IgM was greater than the affinity for pIgA when these immunoglobulins were in the solid phase.

This meant that the presence of IgM in serum samples would interfere with the measurement of pIgA antibody. The original concept that serum could be incubated in antigen-coated wells and the bound pIgA antibody left in the wells after washing be detected by adding labelled SC was no longer feasible as SC

would detect bound IgM antibody too.

To prevent this interference by IgM, measures to relatively inhibit the binding of labelled SC to IgM were explored. As these proved futile it became necessary to deplete serum samples of IgM, before performing the assay for pIgA antibody. The attempt to inhibit the binding of SC to IgM and the method used to remove the IgM from serum are described.

1. Attempts To Selectively Inhibit Secretory Component Binding To IgM But Not Polymeric IgA

Experiments were performed where the conditions under which the assays were performed were altered in an attempt to inhibit the binding of radiolabelled SC to IgM without affecting the binding of SC to pIgA.

In view of the experimental evidence showing that IgM-SC complexes are held together primarily by non covalent forces in contrast to the pIgA-SC complexes which are held together by covalent bonds (Weicker and Underdown, 1975; Brandtzaeg, 1977), attempts were made to interfere with the IgM-SC binding by:

(a) altering the pH of the solution in which SC was added

(b) altering the molarity of the solution in which SC was added

(c) the addition of detergent (Tween) to the solution containing the SC.

These three aspects were examined in the same assay. The effect of adding SC diluted in these solutions was compared to the binding of SC diluted in the usual diluent (BSA-PBS).

Micro titre plates were coated with IgM, blocked and washed as described. SC was incubated in the wells for 4 hours at $4^{o}C$. The plates were washed and the radioactivity bound to the wells measured.

(a) <u>The Effect Of Diluting SC In Buffers Of pH3, pH5, pH6, pH8, pH9 and pH10</u>

A citrate buffer (0.1M disodium citrate) was used for the pH range 3 to 6. (0.1N HCl for pH3; 0.1N NaOH for pH 5 and pH6) and a borate buffer (0.1M Boric acid in 0.1M HCl; + 0.1NaOH) provide the buffers of 8 to 10.

The binding of SC to IgM was inhibited by a solution of pH 5 or less.

(b) <u>The Effect Of Diluting SC In Buffers Of Varying Molarity</u>

The SC was also diluted in solutions of 0.5% BSA-PBS containing 0.5M sodium chloride, 1M

sodium chloride or 2M sodium chloride.

The binding of SC to IgM was inhibited by the solution containing 1M sodium chloride and 2M sodium chloride. 0.5M sodium chloride had no effect.

(c) The Effect Of Detergent

0.5% Tween 20 was added to the 0.5% BSA PBS diluent containing SC. This had no effect on SC binding to IgM.

Therefore this experiment demonstrated that the SC IgM binding could be prevented by adding SC diluted in a solution of pH 5 or less or in a solution containing 1M or 2M sodium chloride.

Having determined the best conditions to use to inhibit the SC-IgM interaction, an experiment was performed to compare the binding of radiolabelled SC to plates coated with pIgA or IgM when SC was diluted in:

(a) a solution of pH 4 or 5
(b) a solution containing 1M sodium chloride
(c) a solution containing PBS and 0.05% Tween 20
(d) 0.5% BSA PBS.

The binding of SC to pIgA and IgM was unaffected by the addition of Tween. The binding of SC to IgM remained stronger than the binding

to pIgA. The solution of low pH and the solution containing 1M sodium chloride inhibited the binding of SC to both pIgA and IgM. The effect on the binding to pIgA is shown (Figure 3.9).

(d) Attempts to Prevent The Binding Of Secretory Component To IgM Using 2-Mercapto Ethanol (BME)

The non covalent forces involved in the polymeric immunoglobulin-SC interactions are much stronger for IgM than pIgA (Brandtzaeg, 1977; Weicker and Underdown, 1975). Therefore an attempt to prevent binding of SC to IgM using 2-mercapto ethanol was made.

Four micro titre plates were coated with pIgA and IgM. The binding of the immunoglobulins to the plates was confirmed by ELISA using one of the plates. The second plate was used to perform the standard assay adding 10ng ^{125}I labelled SC diluted in BSA-PBS to each well. To the third plate 100ul of a 50mM solution of 2 mercapto ethanol (BME) was added per well. After a 1 hour incubation at 37^{o}C the plate was washed and then 10ng labelled SC added per well. The 4th plate was blocked and washed as usual. Then 10ng of ^{125}I labelled SC was added diluted in BSA-PBS containing 50mM BME.

The total number of washes performed before the addition of SC was the same for the 3 plates in the RIA. SC was incubated in the wells for 4 hours at 4°C before the plates were washed and the radioactivity bound measured.

The 2 mercapto ethanol disrupted the binding of SC to pIgA as well as the binding of SC to IgM (Figure 3.10).

Therefore all these attempts to selectively prevent the binding of SC to IgM failed.

2. THE REMOVAL OF IgM FROM SERUM USING ANTI-IgM SEPHAROSE AFFINITY COLUMNS

It became necessary to remove IgM from serum prior to performing the assay for pIgA antibody.

This was done by affinity chromatography.

(a) Construction Of Anti IgM Sepharose Affinity Columns

IgM specific antibody was prepared as described (Chapter 2, section lII,l) and coupled to cyanogen bromide activated Sepharose 4B (Pharmacia). The coupling procedure has been described (Chapter 2, section III,3).

Multiple columns (Amicon C/N Af25) each comprising 2ml of anti IgM Sepharose (lmg

antibody per ml gel) were prepared.

(b) Use Of The Anti IgM Sepharose Affinity Columns

Each 2ml anti IgM column was equilibrated with 0.1M phosphate buffer pH 7.6 (Na_2 HPO_4 KH_2 PO_4) prior to the application of serum. 500ul aliquots of serum were run into each column. After a 40 minute incubation at room temperature unadsorbed proteins were eluted by washing the column with phosphate buffer and collected in essentially 100% yield in the first 5ml (giving a 10 fold dilution of the original sample). The bound protein (IgM) was eluted with 8mls of 3M sodium thiocyanate. The columns were washed extensively with phosphate buffer before they were reused.

(c) The Efficiency Of The Affinity Columns

The ability of the affinity columns to totally deplete even 1ml aliquots of serum of IgM was shown by ELISA and single radial immunodiffusion (in Chapter 2, section III,4).

(d) Specificity Of The Anti IgM Sepharose Affinity Columns

The thiocyanate eluate off the column was dialysed extensively against PBS. Analysis by

ELISA revealed that it contained IgM but no IgG or IgA.

Using an ELISA it was found that the unadsorbed protein was eluted in the first 5ml collected when the columns were washed in phosphate buffer (giving a 10 fold dilution of the original sample).

When 200ug of purified pIgA in 500ul of phosphate buffer was sent through the columns it was virtually all retreived (98.5%) in the first 5ml of the phosphate eluate.

These columns proved to be highly efficient in depleting serum of IgM. In order to be absolutely certain, in all subsequent assays for antigen specific pIgA antibody and total serum pIgA, 500ul aliquots of serum were depleted of IgM by using 2ml anti IgM Sepharose columns. The success of the IgM removal was always confirmed by ELISA and single radial immunodiffusion.

V. OPTIMISING OTHER STEPS IN THE ASSAY

In attempts to optimise the assay, the number of washes performed after the incubation of SC, the length of the incubation period, the amount of SC added per well and the temperature at which the SC was incubated in the wells was examined.

1. The Effect Of The Number Of Washes On SC Binding

The effect of performing 2, 5 and 10 washes with PBS Tween after incubating 10ng of labelled SC in wells coated with pIgA was examined.

There was a decline in the radioactivity bound from 2 to 5 washes (35%) but little difference between 5 and 10 washes (Figure 3.11). The background binding to wells that had no immunoglobulin coating was more acceptable after 5 washes. (Background 370cpm, under 150cpm for 2 and 5 washes respectively). Therefore the plates were always washed 6 times after SC incubation.

2. The Effect Of The Temperature Of The Washing Solution And Presence Of Tween

The use of PBS alone at room temperature or $4^{o}C$ for washing was compared with the use of PBS Tween at room temperature and $4^{o}C$. There was no significant difference.

Therefore washing was always performed with PBS Tween at room temperature.

3. Optimising The Quantity Of SC Added To Each Well

The binding of labelled SC to pIgA and IgM coated wells which occurred with the addition of 40, 20, 10,

5 or 2.5ng of SC diluted in 0.5% BSA PBS was studied. The assay was performed under the usual conditions allowing the SC to remain in the immunoglobulin coated wells for 24 hours at 4°C before washing the plates 6 times with PBS Tween.

The percentage of the counts added per well which bound to pIgA coated wellS with the different concentrations of SC are shown in Figure 3.12.

The percentage of counts added which bound decreased if more than 10ng of SC were added per well (Figure 3.12). The background binding was also increased. Therefore in all subsequent assays 10ng of labelled SC was added to each well.

4. The Optimum Conditions For The Incubation Of SC With pIgA

The temperature and length of time 10ng of labelled SC was incubated in wells coated with pIgA was varied in the next series of experiments. The following incubation conditions were evaluated

(1) 4 hours at 4°C.

(2) 24 hours at 4°C.

(3) 4 hours at 37°C, then 20 hours at 4°C.

(4) 48 hours at 4°C.

Leaving the SC for 24 hours at 4°C improved the binding. There was no advantage in having an initial 4 hour incubation at 37°C or prolonging the incubation period to 48 hours (Figure 3.13).

VI. DETERMINING THE SENSITIVITY OF THE ASSAY

The sensitivity of the assay was determined by radiolabelling pIgA with ^{125}I (Chapter 2, section II, 3b) and using the enzyme linked anti-IgA conjugate to compare the binding of labelled and unlabelled pIgA to the wells of microtitre plates. The binding of radiolabelled SC to unlabelled pIgA was measured.

The radiolabelled and unlabelled pIgA were each diluted to a concentration of 10ug/ml in sodium carbonate coating buffer and serial doubling dilutions were made in a diluent comprising 10ug/ml IgG in coating buffer. The IgG was used as a carrier to minimise adsorptive losses at the low concentrations used and to provide a consistent coating concentration of protein with decreasing levels of pIgA.

Three microtitre plates were used in this experiment. The first plate was used to determine the binding of radiolabelled pIgA to wells. The second for

the ELISA comparing the binding of labelled and unlabelled pIgA to wells. The third plate was coated with unlabelled pIgA and used to measure the binding of labelled SC to pIgA.

The labelled and unlabelled pIgA was left to coat the wells at 4°C overnight. 100ul serial dilutions were incubated in each well. The control wells had no immunoglobulins. The wells were emptied and 150ul of 0.5% BSA-PBS left in them for 45 minutes at room temperature.

The first plate was then washed 12 times with PBS Tween. Residual radiolabelled pIgA bound to the plate was measured.

The second plate was washed 6 times. Then 100ul of goat anti human IgA coupled to alkaline phosphatase (Kirkegaard Perry Laboratories) was added at a dilution of 1/2000 to each well. After a 4 hour incubation at 37°C, the wells were emptied and washed 6 times with PBS Tween. p-Nitrophenylphosphate substrate (1 mg/ml in 10% diethanolamine buffer, pH 9.6) was added, 100ul per well. The OD_{405} produced at 30 mins and 1 hour was read.

The third plate was washed 6 times, then 10ng of ^{125}I labelled SC in 100ul of 0.5% BSA-PBS was added per well. After a 24 hour incubation at $4^{o}C$, the wells were emptied, the plates were washed 6 times and the bound radioactivity was measured.

The binding of radiolabelled pIgA to plate 1 is shown (Figure 3.14).

The ELISA performed using plate 2 showed that there was no difference between the binding of labelled and unlabelled pIgA to the plate (Figure 3.15) confirming that no loss of pIgA had occurred during the labelling procedure and confirming that the concentration of labelled pIgA used to coat the plates was correct.

Radiolabelled SC bound to unlabelled pIgA coated wells as shown (Figure 3.16).

The specific activity of the labelled SC on the day this assay was performed was 6.96×10^3 cpm/ng. The specific activity of the labelled pIgA was 5.42×10^3 cpm/ng. The ELISA showed that there was no difference between the binding of labelled and unlabelled pIgA to the plate (Figure 3.15). Knowing the quantity of radiolabelled pIgA bound, an OD_{405} of

0.15 at 1 hour represented 2ng of pIgA bound to the plates.

Knowing the specific activity of the labelled pIgA was 5.42×10^3 cpm/ng and having measured the radioactivity bound to plate 1 (Figure 3.14) the quantity of pIgA bound to the wells was calculated. A figure depicting the binding of radiolabelled SC to the exact quantity of pIgA bound to the wells was then constructed (Figure 3.17).

The estimation of the degree of binding of SC to pIgA and the determination of the sensitivity of the assay were made using the results expressed in this figure.

Looking at the steep part of the curve (Figure 3.17) where 50ng of pIgA were bound to the wells, the counts per minute bound of SC = 2035 cpm.

As the specific acitivity of the labelled SC was 6.96×10^3 cpm/ng, 2035 cpm represent 0.29 ng SC.

The number of moles of SC bound

$$= \frac{0.29}{75,000} \text{ (taking the MW of SC to be 75,000)}$$

The number of moles of pIgA bound to the plate

$= \frac{50}{335,000}$ (taking the MW of pIgA to be 335,000)

Therefore $\frac{50}{335,000}$ moles of pIgA bound to the plate

captured $\frac{0.29}{75,000}$ moles of SC or 40 moles of pIgA

was required to bind 1 mole of SC.

Considering the application of the assay to detect antigen specific pIgA antibody in serum an amount of bound radioactivity (minus background) which was equivalent to 4 times the background binding (to control wells) was chosen as the end point.

This represented 600cpm of bound SC. Thus at the end point chosen the assay was capable of detecting 20ng pIgA bound to a well (estimated using Figure 3.17).

VII. STABILITY OF THE RADIOLABELLED SECRETORY COMPONENT

The radiolabelled SC had a limited shelf life. In addition to the usual radioactivity decay the

affinity of the labelled SC for IgM and pIgA fell with time. The radiolabelled SC could not be used after 4 weeks of storage at $4^{o}C$.

Considering the binding of the same labelled SC to identical immunoglobulin preparations of pIgA and IgM bound to wells on the day of radiolabelling 3 weeks and 41 days later the percentage of the radioactivity added which bound to pIgA fell from 12% at labelling to 4% 3 weeks later to only 1.9%, 44 days after radiolabelling.

VIII **SUMMARY TO CHAPTER 3**

In this chapter the experiments performed to assess the ability of radiolabelled SC to bind to immunoglobulins bound to the wells of micro titre plates have been described. Radiolabelled SC bound to pIgA and IgM but not to IgG or monomeric IgA or secretory IgA when these immunoglobulins were used to coat the plates.

Having demonstrated that pIgA bound SC, the capacity of soluble pIgA to inhibit the binding of SC to plates coated with pIgA or IgM was demonstrated. Both pIgA and IgM were inhibitory, pIgA being more effective than IgM in inhibiting the binding in contrast to their binding of SC when attached to

plates. Other forms of IgA (monomeric and secretory IgA) and IgG gave no inhibition. The more efficient inhibition of binding of SC to IgM coated wells in the liquid phase may be due to covalent stabilisation of the pIgA-SC complexes by disulphide exchange (Brandtzaeg, 1977). This aspect was not investigated.

To demonstrate that the SC was indeed binding to pIgA on the coating trays and not to IgM which might have been present as a trace contaminant, antibodies specific for IgA and IgM were each tested for their capacity to block the SC binding. Exposure of pIgA coated wells to anti IgA antibodies totally inhibited the binding of SC. In contrast exposure of the pIgA coated wells to anti IgM antibodies did not inhibit the binding of SC.

The specificity of the SC binding assay for the detection of antigen specific pIgA was confirmed by fractionating IgM depleted serum samples on Sephacryl S200 and showing that the SC binding activity was associated with fractions containing pIgA and not in fractions containing monomeric IgA. The SC binding activity of the IgM depleted sera was removed by passage through an anti IgA sepharose column confirming that the binding activity was due to pIgA antibodies.

The affinity of SC for solid phase bound IgM was greater than for pIgA. This is consistent with the findings of other investigators (Brandtzaeg, 1977; Socken and Underdown, 1978). Several attempts to inhibit the binding of labelled SC to IgM without causing interference to the binding of SC to pIgA were unsuccessful. The use of 2ml anti IgM Sepharose columns to deplete serum samples of IgM proved to be effective.

The affinity of the labelled SC for pIgA was low, 12% of counts added to each well bound to solid phase pIgA. The experiments performed to optimise the assay are described. The sensitivity of the assay was determined choosing the end point which represented 20ng of bound pIgA.

The assay was successfully used to detect antigen specific pIgA antibody in human serum (See Chapters 6 to 8).

In view of the low affinity of labelled SC for pIgA several strategies to improve the sensitivity of the radioimmunoassay were explored and as the radiolabelled SC had a limited shelf life attempts to substitute the radioimmunoassay with an enzyme linked

immunosorbent assay were made. These strategies are described in the next chapter.

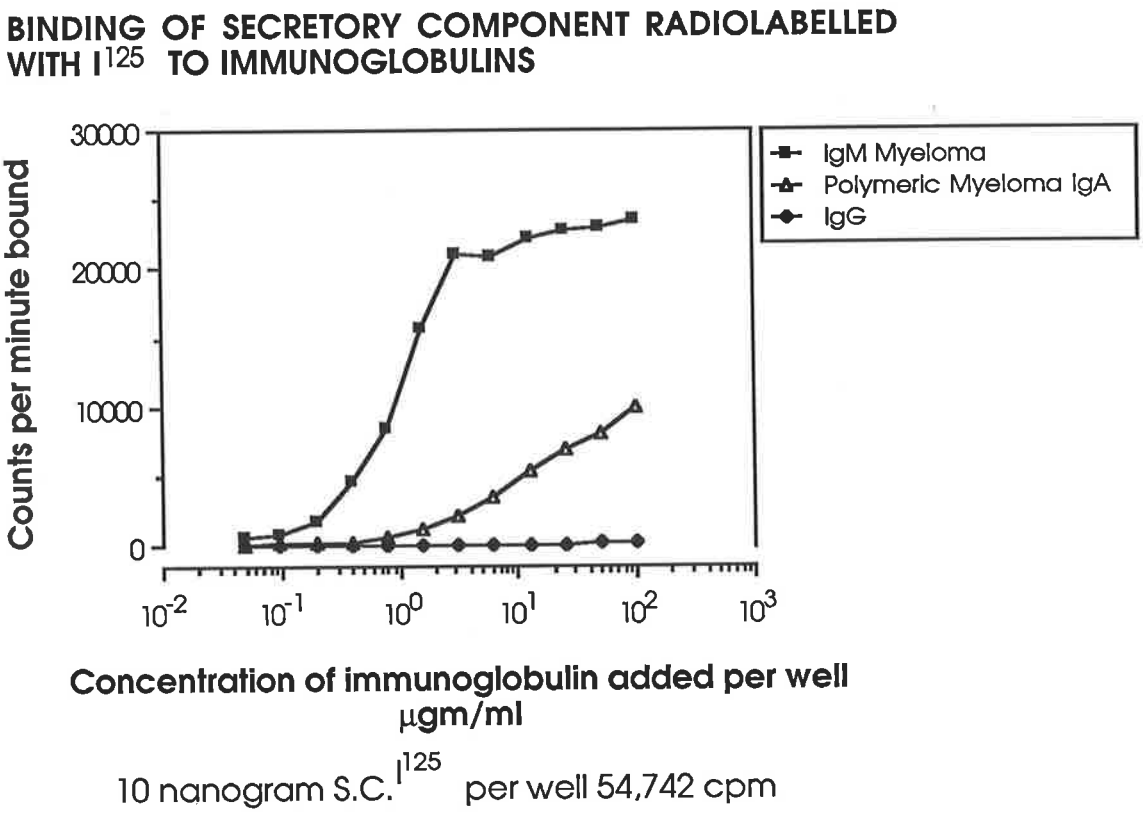


Figure 3.1

The binding of ^{125}I labelled SC (10ng) to solid phase immunoglobulins (myeloma IgM, polymeric myeloma IgA, IgG). Plates were coated with different concentrations of each immunoglobulin as indicated, before blocking and addition of labelled SC.

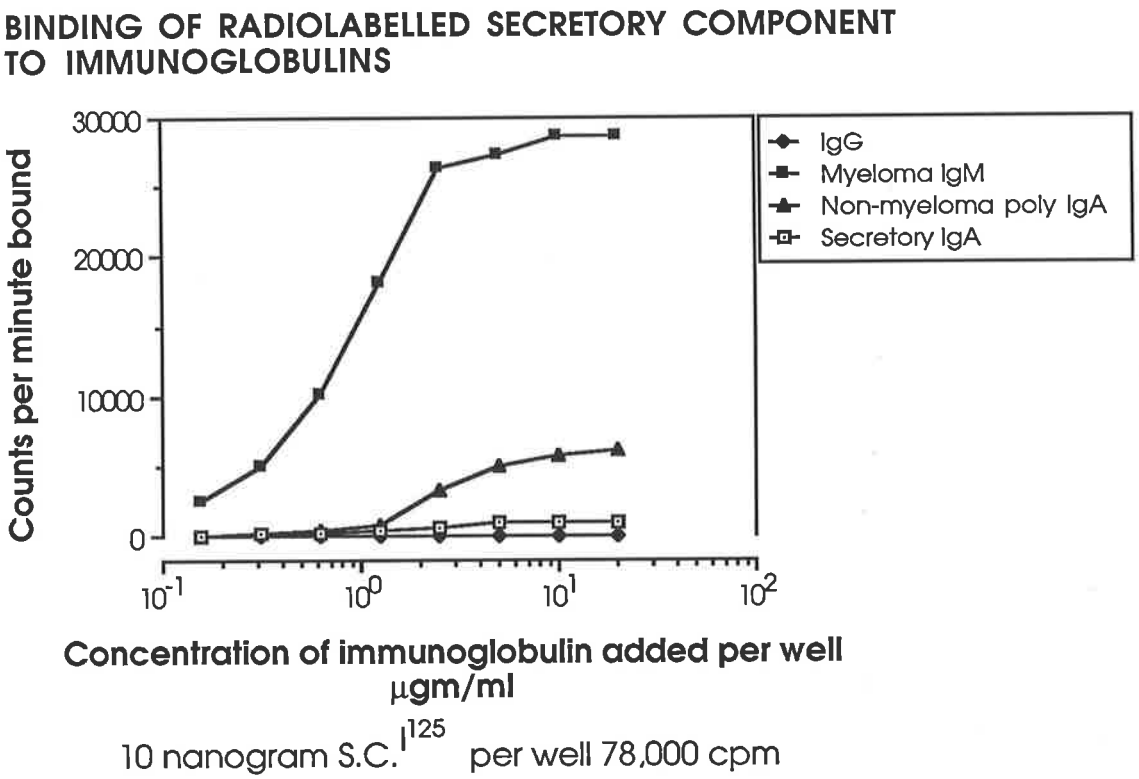


Figure 3.2

The binding of ^{125}I labelled SC (10ng) to solid phase immunoglobulins (IgG, myeloma IgM, non-myeloma pIgA and secretory IgA). Plates were coated with different concentrations of each immunoglobulin as indicated, before blocking and addition of labelled SC.

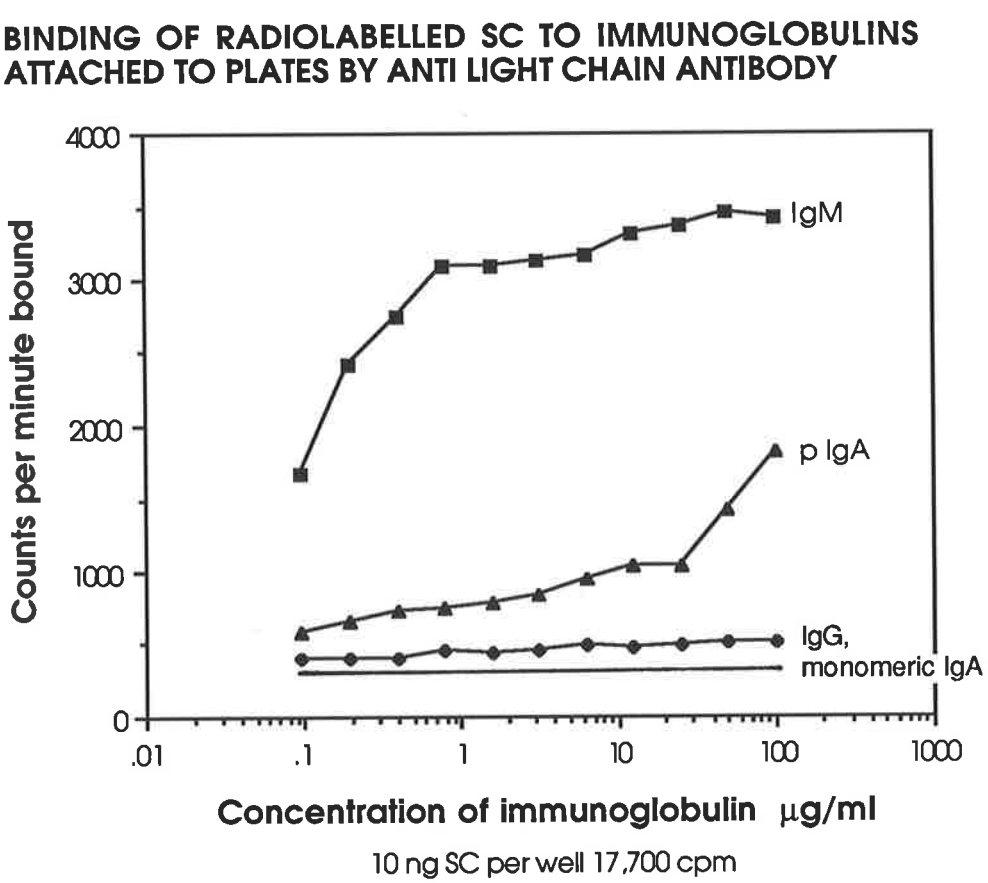
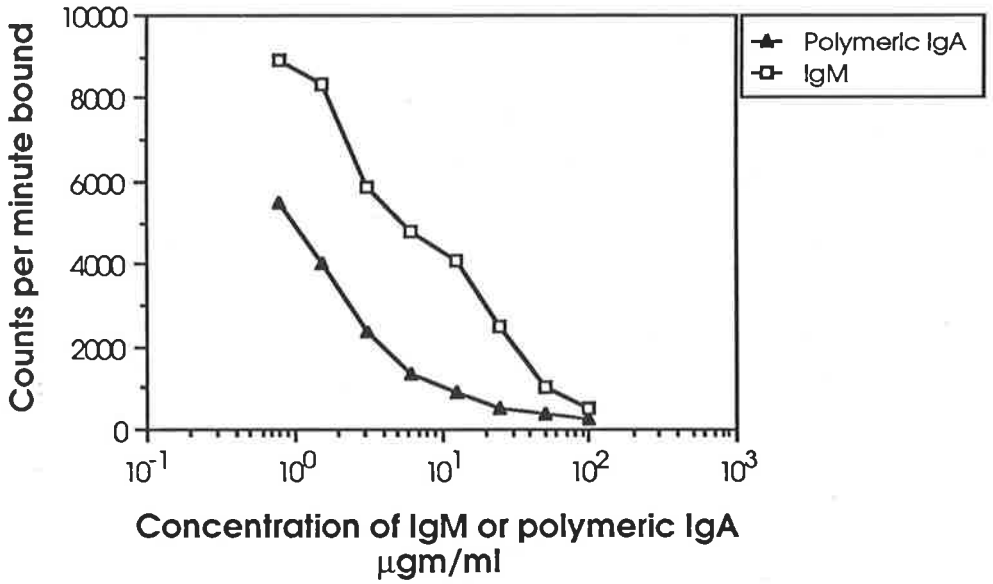


Figure 3.3

The binding of ^{125}I labelled SC (10ng) to immunoglobulins (IgM, pIgA, IgG and monomeric IgA) attached to plates through their binding to anti light chain antibody. The plates were coated with 10ug/ml anti light chain antibody. After blocking and washing different concentrations of each immunoglobulin were incubated in the wells as indicated. The plates were washed before the addition of labelled SC.

INHIBITION OF BINDING OF SECRETORY COMPONENT TO POLYMERIC IgA BY POLYMERIC IgA OR IgM



Uninhibited binding SC to polymeric IgA = 15,000 cpm

Figure 3.4

The capacity of pIgA or IgM to inhibit the binding of labelled SC to solid phase pIgA. Plates were coated with pIgA (100ug/ml). After blocking, a range of dilutions of pIgA or IgM were added to and mixed with the labelled SC in the pIgA coated wells. Uninhibited binding to pIgA was 15,000cpm.

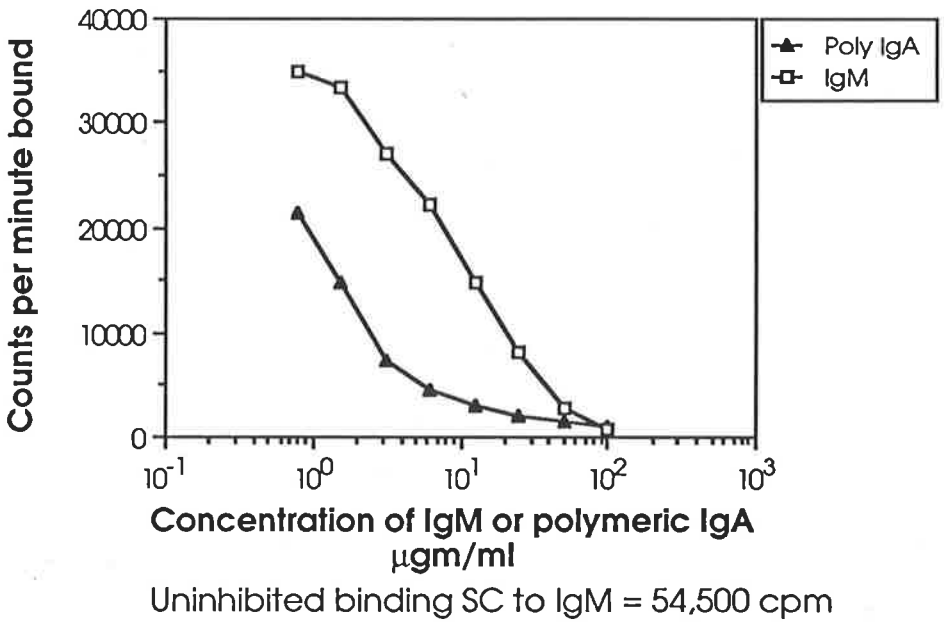


Figure 3.5

The capacity of pIgA or IgM to inhibit the binding of labelled SC to solid phase IgM. Plates were coated with IgM (10ug/ml). After blocking, a range of dilutions of pIgA or IgM were added to and mixed with the labelled SC in the IgM coated wells. Uninhibited binding to IgM was 54,000cpm.

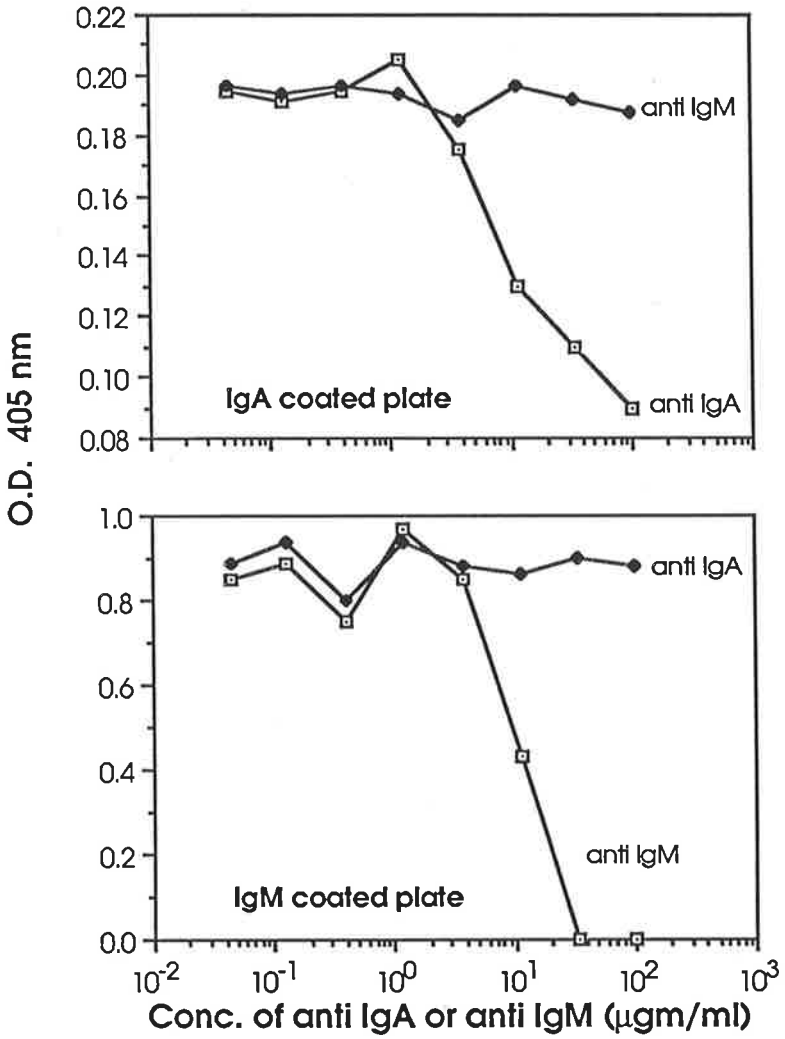


Figure 3.6

The capacity of antibodies specific for IgA or IgM to block the binding of SC to solid phase pIgA or IgM. Both antibodies were purified from polyclonal antisera. SC binding was assessed by ELISA using alkaline phosphatase conjugated to a monoclonal antibody specific for SC.

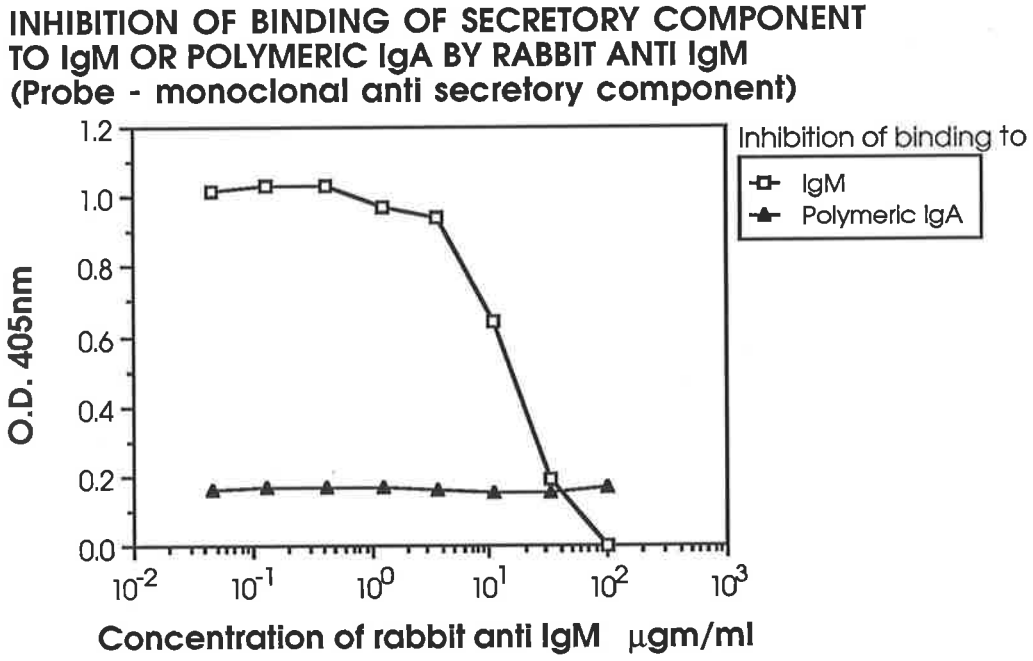


Figure 3.7

The capacity of anti IgM antibody to block the binding of SC to solid phase pIgA or IgM. SC binding was assessed by ELISA using the monoclonal anti SC antibody.

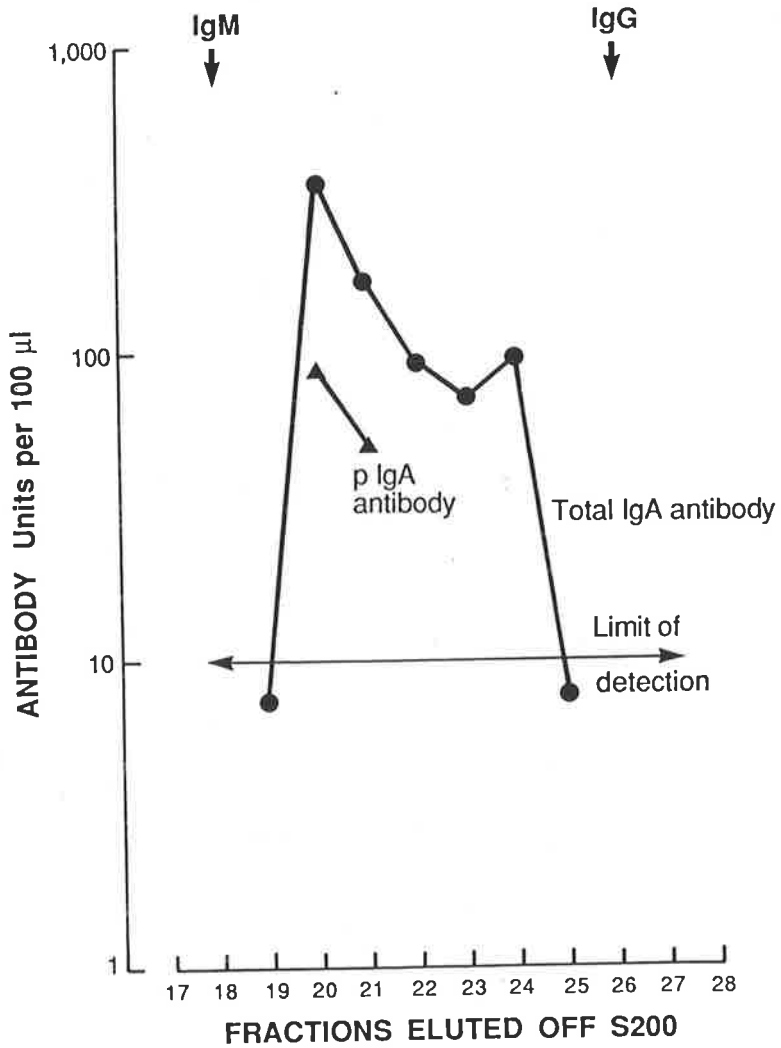


Figure 3.8

IgA antibody to gliadin in fractions of an IgM depleted serum sample of a patient with coeliac disease eluted off an Sephacryl S200 column.

^{125}I labelled SC was used to detect pIgA antibody to gliadin and total IgA antibody to gliadin (monomeric and pIgA antibody) was detected using an ELISA.

pIgA antibody was only detected in fractions 20 and 21.

The fractions where purified IgM and IgG standards would be found after gel chromatography on the S200 column are indicated.

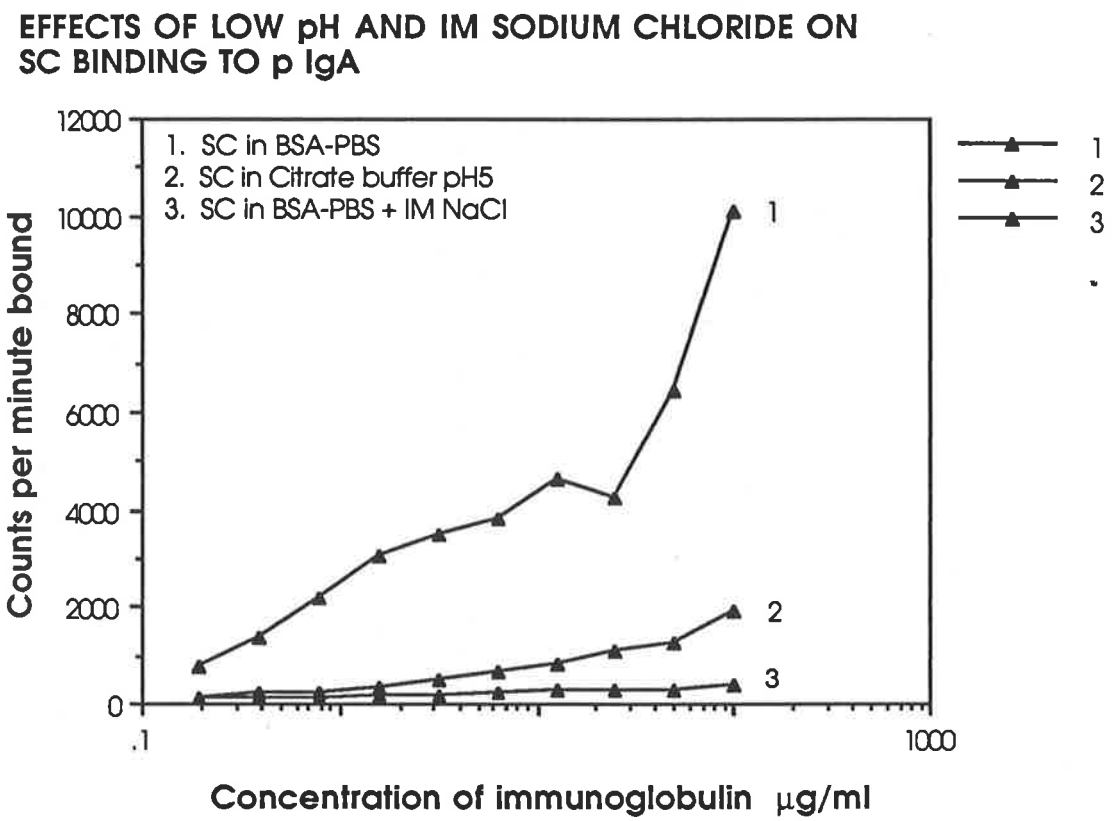


Figure 3.9

The binding of ^{125}I labelled SC to solid phase pIgA when SC was diluted in:

1. BSA-PBS
2. Citrate buffer pH5
3. BSA-PBS containing lM NaCl

The plate was coated with different concentrations of pIgA as indicated before blocking and the addition of labelled SC.

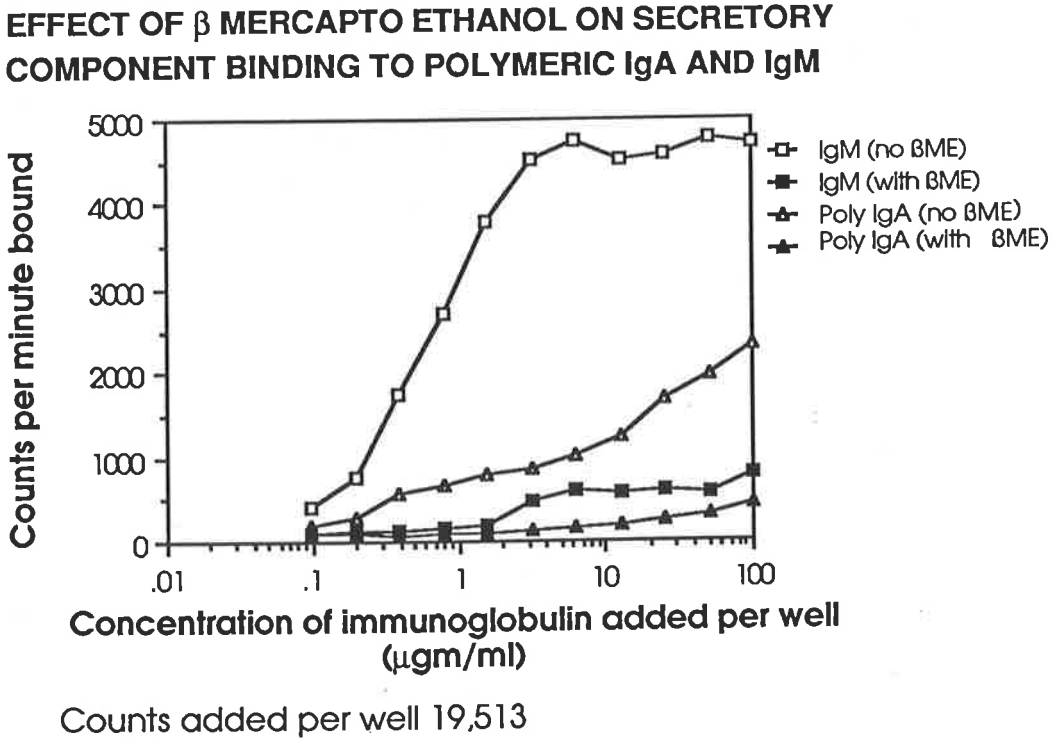


Figure 3.10

Attempts to selectively inhibit the binding of ^{125}I labelled SC to solid phase IgM without affecting the binding of SC to pIgA using 2-mercapto ethanol (BME). Plates were coated with different concentrations of each immunoglobulin as indicated. After blocking and washing labelled SC was added diluted in BSA-PBS or in BSA-PBS containing 50mM BME.

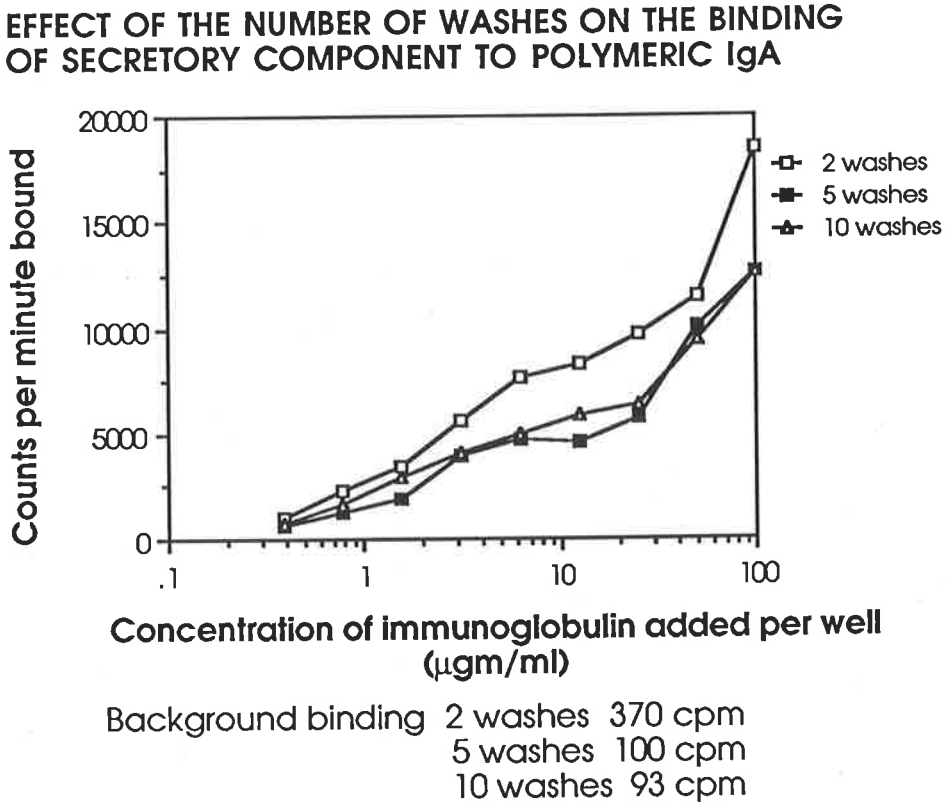
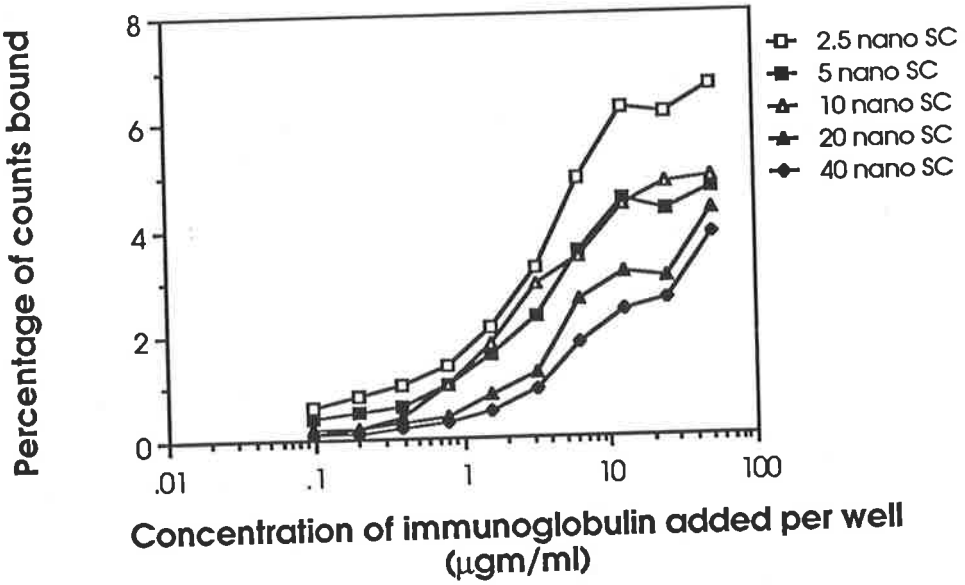


Figure 3.11

The effect of the number of washes performed after the incubation of labelled SC in wells coated with pIgA, on the binding of SC to pIgA. Plates were coated with different concentrations of pIgA as indicated, 10ng SC was added per well. The radioactivity bound to the wells is indicated as well as the background binding of ^{125}I SC to wells which had not been coated with pIgA.

THE PERCENTAGE OF THE RADIOLABELLED SECRETORY COMPONENT ADDED TO POLYMERIC IgA COATED WELLS WHICH WERE BOUND WHEN DIFFERENT CONCENTRATIONS OF S.C. WERE ADDED



Counts added per well
40 nano SC = 252,025
20 nano SC = 127,999
10 nano SC = 63,359
5 nano SC = 33,118
2.5 nano SC = 17,069

Figure 3.12

The binding of ^{125}I labelled SC to pIgA with the addition of

1. 2.5ng SC
2. 5ng SC
3. 10ng SC
4. 20ng SC
5. 40ng SC

The concentration of pIgA used to coat the well is shown. The percentage of the counts added to each well which bound to the well are indicated.

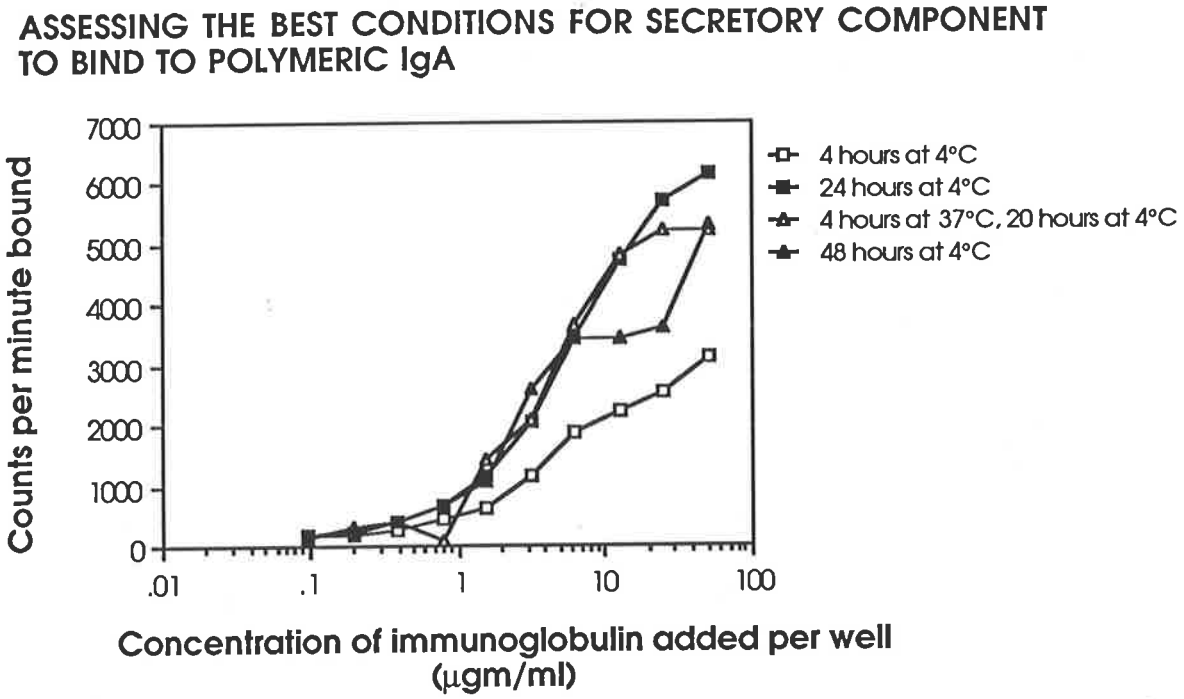


Figure 3.13

The effect on the binding of ^{125}I labelled SC to solid phase pIgA when SC was incuated in the wells for

1. 4 hours at $4^{o}C$
2. 24 hours at $4^{o}C$
3. 4 hours at $37^{o}C$ followed by 20 hours at $4^{o}C$
4. 48 hours at $4^{o}C$

10ng ^{125}I labelled SC was added per well. The concentration of pIgA used to coat the wells is shown.

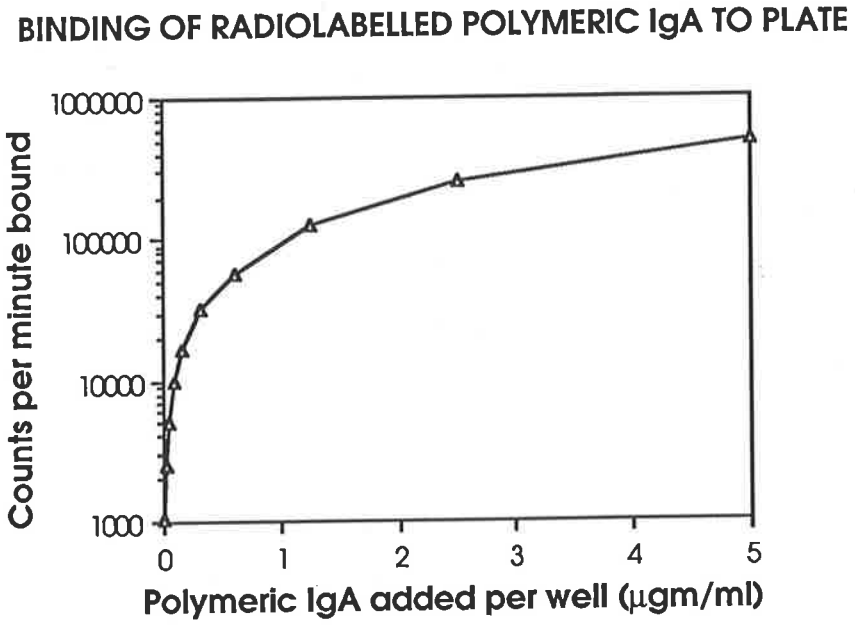


Figure 3.14

The binding of ^{125}I labelled pIgA to microtitre plates. The radioactivity bound to the plate after overnight incubation of pIgA in the wells and washing is shown. The concentrations of pIgA added per well is indicated.

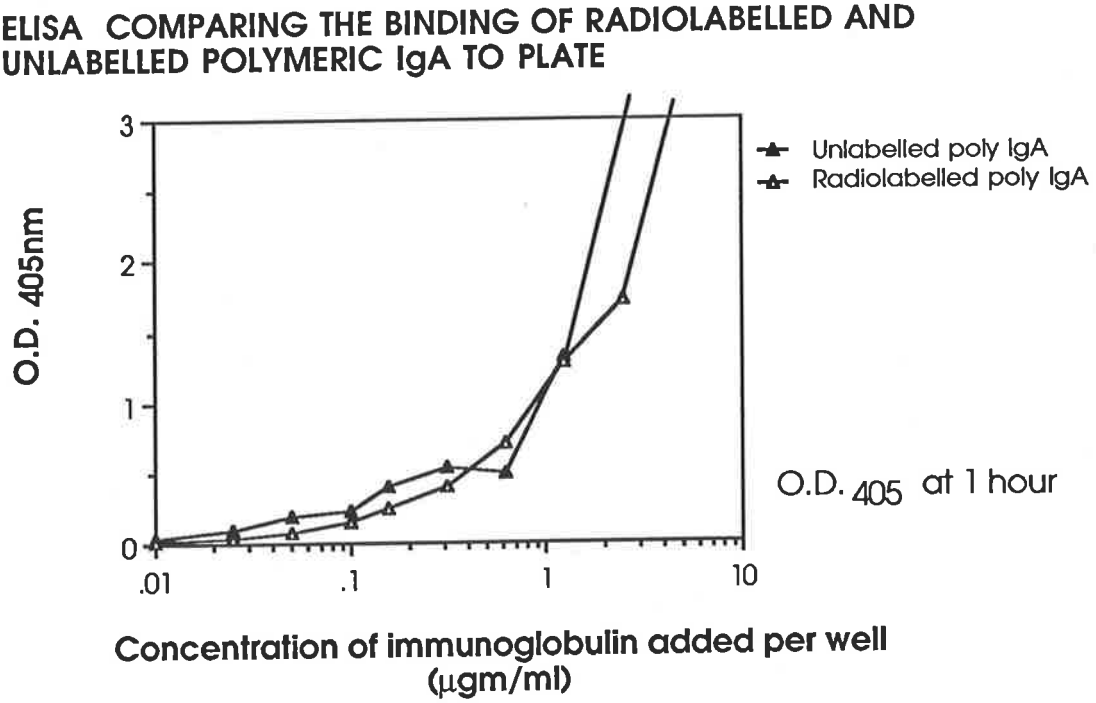


Figure 3.15

The binding of pIgA labelled with ^{125}I and unlabelled pIgA to microtitre plates. The binding was measured by ELISA using alkaline phosphatase coupled to heavy chain specific anti IgA antibody. The concentration of pIgA added per well is indicated and the OD_{405} measured after incubation of the substrate for 1 hour is shown.

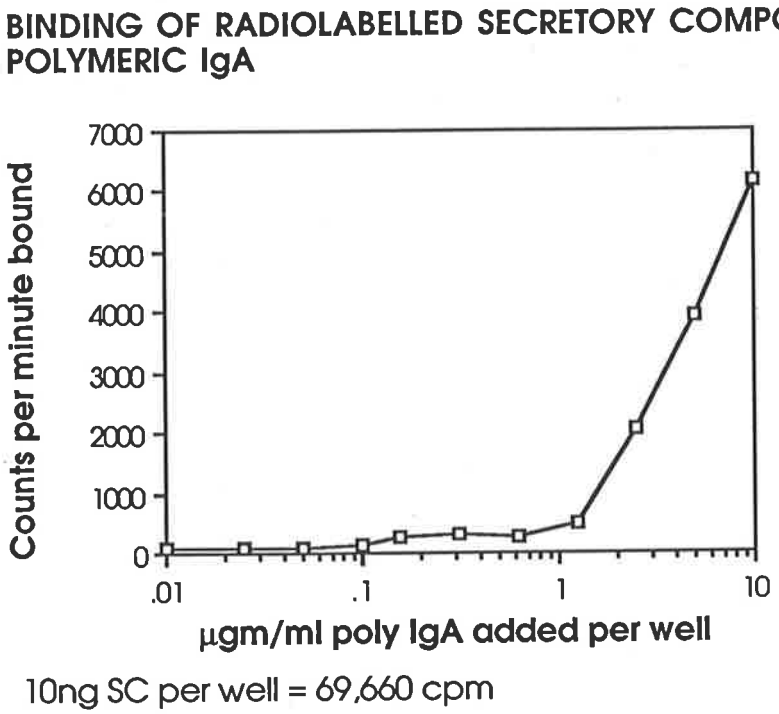


Figure 3.16

The binding of ^{125}I labelled SC to pIgA bound to plates. The concentration of pIgA added per well is shown.

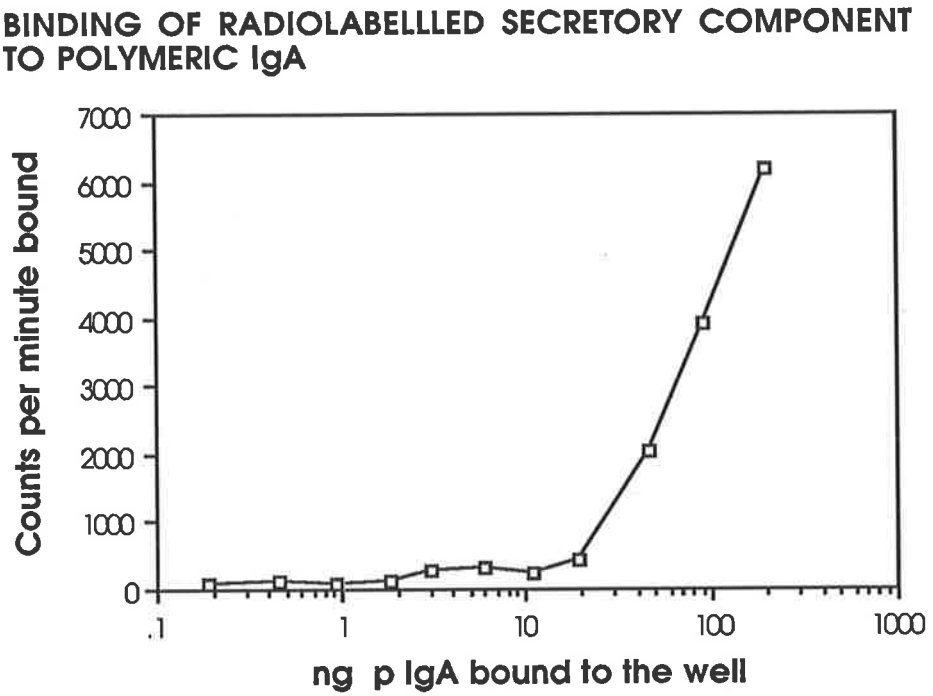


Figure 3.17

The binding of ^{125}I labelled SC to pIgA bound to micro titre plates. The actual concentration of pIgA bound to the wells is shown. At the end point chosen (4 times the background i.e. the binding of ^{125}I labelled SC to wells in which no pIgA had been incubated) where 600cpm was bound, 20ng of pIgA was bound to the wells.

CHAPTER 4 - STRATEGIES EMPLOYED IN AN ATTEMPT TO INCREASE THE SENSITIVITY OF THE ASSAY FOR POLYMERIC IgA ANTIBODY

1. INTRODUCTION

The radioimmunoassay described in Chapter 3 could detect 20ng of solid phase pIgA. It was successfully used to detect antigen specific pIgA antibody in healthy volunteers and patients (Chapters 6,7,8). However, it was apparent from the relative molar concentrations of SC and pIgA (Chapter 3, section VI) that the radiolabelled SC was behaving suboptimally as 1 molecule of SC did not bind 1 molecule of pIgA.

This chapter describes experiments performed and strategies employed in an attempt to increase the sensitivity of the assay for pIgA antibody.

The SC that was radiolabelled was purified using anion exchange and gel chromatography and judged to be free of contamination with immunoglobulins or lactoferrin by immunodiffusion, immunoassay and polyacrylamide gel electrophoresis. Although free of contaminants the manipulation of SC may have affected its ability to bind pIgA. Therefore an alternative method of preparing SC using IgM sepharose affinity chromatography which had the potential benefit of selecting a "functional" SC was examined and the

ability of this SC radiolabelled with ^{125}I to bind pIgA evaluated.

Some proteins are altered by the process used to radioiodinate them (Bolton and Hunter, 1973). Therefore alternative methods of radiolabelling SC were examined. These attempts to improve the RIA are described in Section II.

In an attempt to avoid the effects of iodination and the problem of long term stability of ^{125}I labelled SC (Chapter 3, section VII) the substitution of the radioimmunoassay with an enzyme linked immunosorbent assay was attempted. Two approaches were explored: firstly the use of an enzyme linked SC (Section III,1) and secondly the use of an enzyme linked monoclonal anti SC antibody to detect the binding of "free" unlabelled SC to pIgA (Section III,2). This ELISA was also used to study the effect of radioiodination on the binding of SC to pIgA.

II. STUDIES OF ALTERNATIVE METHODS OF PREPARING AND LABELLING SECRETORY COMPONENT FOR USE IN THE RADIOIMMUNOASSAY

1. The Use Of Secretory Component Prepared By Affinity Purification Using IgM Sepharose

In view of the possibility that the SC was

damaged during the purification an alternative method of preparing SC was evaluated. Rather than using anion exchange chromatography (as described in Chapter 2, section II,1,a(i), SC was prepared from human colostrum by affinity chromatography using IgM sepharose. (Chapter 2, section II,1,a,(ii)) 50ng of the SC obtained by this method was radiolabelled with ^{125}I using the chloramine T method (Chapter 2, section II,1,c,(i)).

The binding of this radiolabelled SC to pIgA, IgM and IgG was examined. The assay was performed as described in Chapter 3, section 1.

Briefly plates were coated with pIgA, IgM or IgG. After blocking and washing, 10ng of ^{125}I labelled SC was incubated in each well for 4 hours at $4^{o}C$. After washing the bound radioactivity was measured.

The binding to IgM was enhanced (Figure 4.1). The peak binding to IgM was approximately 50% of the radioactivity added to each well. In previous assays the peak binding to IgM had been approximately 30% of the radioactivity added.

The binding to pIgA was not improved. The peak binding to pIgA was 6% of the radioactivity added. The binding to IgG was less than background.

This result suggested that the affinity purification selected SC which had a strong affinity for IgM. There did not appear to be any difference between the SC preparations on polyacrylamide gel electrophoresis.

2. The Evaluation Of Secretory Component Radiolabelled By Alternative Methods

The chloramine T procedure of radioiodinating proteins is an efficient method of directly introducing ^{125}I into the tyrosine residue of proteins. However, some proteins are altered during radioiodination and this may result in loss of affinity (Bolton and Hunter, 1973). There is some evidence that the damage is caused by impurities in the ^{125}I (Bolton and Hunter, 1973). Exposure to the oxidising agent (chloramine T) and the reducing agent (sodium metabisulphite) may disrupt the structural integrity of the SC.

Therefore two alternative methods of labelling SC were evaluated.

a) Secretory Component Radiolabelled Using Iodogen Tubes

This method of labelling SC (described in Chapter 2, section II,1,c,(ii)) eliminates the reduction step employed at the close of iodination with soluble chloramides such as chloramine T. The protein is iodinated under milder conditions with the reagents for iodination being in a potentially less destructive solid phase.

SC was radiolabelled with ^{125}I using Iodogen tubes (Chapter 2, section II,1,c,(ii)) and the binding of labelled SC to solid phase immunoglobulins examined.

The results obtained using SC radiolabelled by this method were no different to those obtained initially with SC radiolabelled using chloramine T.

b) Secretory Component Radiolabelled Using The Bolton And Hunter Reagent

The theoretical advantage of the Bolton and Hunter reagent was that the method avoids direct exposure of proteins to ^{125}I. It avoids exposure of the protein to the 3 most likely causes of iodination damage, exposure to oxidising and reducing agents and to the ^{125}I solution.

SC was radiolabelled using the Bolton and Hunter reagent (Chapter 2, section II,1,C,(iii)) and the binding of the labelled SC to solid phase immunoglobulins assessed.

Micro titre plates were coated with serial doubling dilutions of pIgA, IgM, secretory IgA and IgG from a starting concentration of 40ug/ml. After overnight incubation, blocking and washing as described (Chapter 3, section I) 10ng of labelled SC in 100ul BSA PBS (specific activity 1200 counts per ng) was incubated in the wells for 4 hour at $4^{o}C$. The plates were washed 6 times with PBS Tween and the bound radioactivity measured.

The peak binding to IgM was only 520 cpm and to the pIgA 100 cpm. Counting for 10 minutes gave a peak binding of 5200 counts per 10 minutes for IgM (4% of the radioactivity added per well) and 1000 counts per 10 minutes for pIgA (0.8% of the radioactivity added).

This experiment was repeated leaving the labelled SC for 24 hours at $4^{o}C$ and also adding 25ng of labelled SC to each well.

The binding to pIgA and IgM was not improved.

3. Experiments Examining The Effect Of Radioiodination On Secretory Component

The possibility that the SC was damaged during the process of radioiodination was investigated in experiments utilising the alkaline phosphatase labelled anti SC in an ELISA. This ELISA is described in the next section (Section III,2).

a) The Effect Of Chloramine T On Secretory Component

Suspecting that the SC was damaged during the oxidation induced by chloramine T an experiment was performed to test the possibility, where SC was exposed to chloramine T under the identical conditions used in the radiolabelling procedure (Chapter 2, section II,1,c,(i)). However, no ^{125}I was added. The binding of native SC and SC which had been exposed to chloramine T, to IgM coated wells was compared using an ELISA with anti SC (Section III,2).

100ug of SC in 0.1ml of phosphate buffer pH 7.6 was mixed with 0.1ml of chloramine T (0.8mg/ml) which had been freshy prepared. After exposing the SC to the chloramine T for 3 minutes at room temperature 0.1mg of sodium metabisulphite was added. The SC was then mixed with 2ml of 5% BSA PBS.

The wells of micro titre plates were coated with IgM (100ul/well of 10ug/ml in 0.05M sodium carbonate buffer pH 9.6). After overnight incubation at 4°C the plates were blocked with BSA PBS and washed. Duplicate two fold serial dilutions of chloramine T treated SC and untreated SC were incubated in the wells (100ul per well) for 4½ hours at 4°C. After 6 washes with PBS containing 0.05% Tween 20 the alkaline phosphatase labelled anti SC conjugate diluted 1/500 in BSA PBS (Section III,2) was incubated in the wells at room temperature overnight.

The colour (OD_{405} minus background produced by binding of conjugate direct to IgM coated wells) produced after an 8 hour incubation with substrate (Sigma p nitro phenyl phosphate in 10% diethanolamine HCl pH 9.6) at 37°C was read.

There was no difference between the binding of the untreated SC and the SC exposed to chloramine T to the IgM coated wells. The conclusion was that the chloramine T did not by itself have an adverse effect on SC.

b) The Effect Of The Incorporation Of ^{125}I In Secretory Component On SC Binding to Immunoglobulins

An experiment was performed to examine the effect the incorporation of ^{125}I into SC might have on the affinity and binding of SC to polymeric immunoglobulins.

The binding of unlabelled SC and ^{125}I labelled SC to IgM coated wells was compared by ELISA utilising the monoclonal anti SC conjugate to detect SC binding to solid phase IgM. (The detailed conditions for performing the ELISA are described in section III,2).

A micro titre plate was coated with IgM (100ul per well of 5ug/ml). After blocking and washing 100ul of SC in BSA PBS was incubated in each well for 4½ hours at 4°C. Duplicate serial doubling dilutions of unlabelled SC and ^{125}I labelled SC were incubated on the plate.

The unlabelled SC was diluted from a starting dilution of 5ug/ml and the labelled SC from 250ng per ml in view of the radioactivity. After washing, the anti SC conjugate was incubated in the wells overnight at room temperature. The colour (OD_{405} minus background) produced after a 6 hour incubation of the

substrate in the wells was read.

The colour obtained where radiolabelled SC was incubated in the IgM coated well was reduced (see below).

THE BINDING OF LABELLED AND UNLABELLED SC TO SOLID PHASE IgM

Unlabelled SC		Radiolabelled SC	
concentration per well (ng/well)	OD_{405}	concentration per well (ng/well)	OD_{405}
31.25	= 1.108	25	= 0.185
15.6	= 1.034	12.5	= 0.018
7.8	= 0.944	6.25	= below background

e binding of SC was detected using the anti SC conjugate.

This suggested that the incorporation of the ^{125}I into tyrosine residues in SC had a deleterious effect on the binding of the protein to IgM or that it altered the epitope recognised by the enzyme labelled anti SC antibody.

4. SUMMARY

Therefore neither the alternative method of preparing SC or the alternative methods of radiolabelling SC enhanced the affinity of radiolabelled SC for pIgA. The iodination of SC appeared to be responsible for the reduced affinity of SC for pIgA.

III. ENZYME LINKED IMMUNOSORBENT ASSAY SYSTEMS TO MEASURE POLYMERIC IgA ANTIBODY

The limitations of the radioimmunoassay for pIgA are those inherent to most radioimmunoassays. There is the possible damaging effect of the oxidisation and reduction required for iodinating the SC. In addition the ^{125}I itself appeared to damage the SC. Variability of incorporation of the ^{125}I with each radioiodination and the limited shelf life of the labelled SC which could only be used for 4 weeks after radiolabelling (Chapter 3, section VII) were further drawbacks.

The experiments presented in the previous section (Section II) showed that radioiodination reduced the affinity of SC for polymeric immunoglobulin. Attempts to overcome this using a different method to prepare SC and 2 different methods of radiolabelling SC failed to improve the affinity of labelled SC for pIgA.

Therefore the use of an enzyme linked immunosorbent assay system was an attractive alternative approach. Two systems were evaluated. Firstly the use of an enzyme linked SC (with alkaline phosphatase conjugated to SC) and secondly, the use of an enzyme linked monoclonal anti SC antibody which could detect SC bound to pIgA.

The anti SC ELISA was used to validate the results obtained with the radiolabelled SC and also to examine possible reasons for the difficulties experienced with the RIA.

1. The Secretory Component Alkaline Phosphatase Conjugate

The first ELISA system to be evaluated was the use of SC coupled to alkaline phosphatase. SC prepared from human colostrum (Chapter 2, section II,1,a(i)) was coupled to alkaline phosphatase (Sigma, P-5521, Calf intestine Type VII-5) by Mrs. D. DiMatteo

(Chapter 2, section IV,1).

The ability of the SC conjugate to bind to pIgA and IgM bound to the wells of micro titre plates was examined.

Micro titre plates were coated with pIgA and IgM in the usual manner and the binding confirmed by ELISA (Chapter 3, section I). After blocking and washing the SC alkaline phosphatase conjugate was added (100ul per well).

The binding of 4 dilutions of the SC conjugate was examined i.e.

(i) 1/100 =100ng SC per well
(ii) 1/250 = 40ng SC per well
(iii) 1/500 = 20ng SC per well
(iv) 1/1000 = 10ng SC per well

The diluent was 0.5% BSA PBS.

The binding of the SC conjugate to the immunoglobulins was examined under the following conditions.

(i) A 4 hour incubation at 37°C

(ii) A 24 hour incubation at 4°C

After incubating the SC conjugate in the wells the plates were washed. 100ul of substrate (1mg/ml Sigma p nitro phenyl phosphate in 10% diethanolamine-HCl, pH 9.6) was incubated in each well at 37°C. The colour (OD_{405} minus background) produced was measured at intervals up to 24 hours.

No colour change was detected in the wells in which the SC alkaline phosphate conjugate had been incubated even after a 24 hour incubation of the substrate.

It was concluded that the alkaline phosphatase labelled SC conjugate would not bind to solid phase pIgA or IgM

There were several possible reasons for the failure of the SC alkaline phosphatase conjugate assay.

Firstly, the process of conjugating alkaline phosphatase to SC might have damaged the SC.

Secondly, the complex of SC (MW 75 to 80,000) and alkaline phosphatase may have been too large to permit the interaction between SC and the pIgA to take place (steric hindrance).

A third possibility was that the large SC enzyme complex easily dissociated from the pIgA and IgM during the washes performed prior to the addition of substrate.

Nevertheless this attempt to develop an enzyme linked system failed.

2. The Mouse Monoclonal Anti Secretory Component Alkaline Phosphate Conjugate

a) Introduction

The second enzyme linked immunosorbent assay system to be evaluated was the use of a labelled anti SC antibody to detect the binding of "free" SC to pIgA.

This approach had two theoretical advantages over the use of labelled SC. Firstly, it avoided the exposure of SC to potentially damaging reagents. Secondly, it avoided the need for another chemical which could hinder the binding of SC to pIgA to be attached to SC.

A mouse monoclonal anti secretory component antibody was conjugated to alkaline phosphatase (Chapter 2, section IV, 2) and its ability to detect the binding of SC to pIgA evaluated.

b) Description Of The Enzyme Linked Immunoadsorbent Assay For Antigen Specific Polymeric IgA Antibody Using The Anti Secretory Component Conjugate

The wells of micro titre plates (COSTAR) were coated with antigen diluted in 0.05M sodium carbonate, pH 9.6 (100ul per well). After overnight incubation unbound sites were blocked with 0.5% BSA PBS (150ul per well) for 45 minutes at room temperature and then washed with PBS containing 0.05% Tween 20.

IgM depleted serum samples were incubated in a series of doubling dilutions (in BSA PBS) in the wells at 4°C overnight. (100ul per well). The plates were washed 10 times with PBS Tween and then 100ul of 5ug/ml SC in BSA PBS was left in each well at 4°C for 4 hours. After 6 washes with PBS Tween 100ul of a 1/500 dilution of the anti SC conjugate in BSA PBS was left in each well at room temperature overnight. After 6 washes, 100ul of substrate (1mg per ml Sigma p-nitrophenyl phosphate in 10% diethanolamine buffer pH 9.6) was incubated in each well at 37°C and the

colour produced (OD_{405} minus background) at 6 hours read using a Titertek Multiskan ELISA reader (Flow Laboratories).

The background readings were obtained from wells coated with antigen to which no serum samples were added and to which SC, anti SC and substrate were added. The other control wells were those where anti SC and substrate were added to antigen coated wells which had been blocked but to which no serum and no SC was added.

c) Applications Of The ELISA Using Alkaline Phosphatase Labelled Anti Secretory Component

The monoclonal anti SC conjugate was used in a series of experiments. The ELISA was used to validate the results obtained with radiolabelled SC.

(i) ELISA To Demonstrate The Specificity Of The Binding Of Secretory Component To Polymeric IgA And IgM

The specificity of the binding of SC to pIgA and IgM was demonstrated using anti IgA specific antiserum (SILENUS) and anti IgM specific antisera (Chapter 2, section III,1) to inhibit the binding of SC to pIgA and IgM coated micro titre plate. The alkaline phosphatase labelled

anti SC was used to detect the SC bound to the immunoglobulin.

The ELISA was described previously (Chapter 3, section III,1,d).

The anti SC conjugate could detect the binding of SC to pIgA and IgM (Figures 3.6 and 3.7).

Exposure of pIgA coated wells to anti IgA antibody totally inhibited the binding of SC to pIgA and exposure of IgM coated wells to anti IgM antibody totally inhibited the binding of SC to IgM (Figure 3.6).

In contrast exposure of IgM coated wells to saturating amounts of anti IgA and of pIgA coated wells to saturating amounts of anti IgM antibody did not inhibit the binding of SC (Figure 3.6).

This experiment confirmed that the SC bound specifically to pIgA. Once again the binding of SC to IgM was greater than the binding of SC to pIgA (Figures 3.6 and 3.7).

(ii) Application Of The ELISA To Detect Antigen Specific Polymeric IgA Antibody

Having demonstrated the effectiveness of the ELISA in detecting the binding of SC to purified pIgA bound to micro titre plates the effectiveness of the ELISA in detecting antigen

specific pIgA in aliquots of IgM depleted serum was investigated.

The ELISA was used to detect serum pIgA antibody to gliadin in patients with coeliac disease and serum pIgA antibody to typhoid lipopolysaccharide (LPS) in normal volunteers immunised with the oral typhoid vaccine Ty21a.

Measurement Of Polymeric IgA To Gliadin

An attempt to detect serum pIgA antibody to gliadin in the serum of 6 patients with coeliac disease utilising the anti SC ELISA was made. IgM depleted samples which had been demonstrated to contain pIgA antibody to gliadin utilising the ^{125}I labelled SC assay were examined.

Two ELISAs were performed in parallel. The anti SC ELISA was used to detect pIgA antibody to gliadin. An ELISA using commercial heavy chain specific conjugates was used to detect the total IgA antibody to gliadin and the IgM antibody to gliadin. Assays were performed on samples before and after IgM depletion.

The wells of micro titre plates were coated with 5ug/ml Sigma gliadin (as described in Chapter 2, section V,3,a,(iii)). After washing serial doubling dilutions of the samples were incubated in the wells (100ul per well) at $4^{o}C$

for 4 hours. The plates were then washed with PBS Tween.

The assay for pIgA to gliadin using SC and then the anti SC conjugate was performed as described (Chapter 4, section III,2,b). The ELISA for IgA antibody and IgM antibody was performed as described previously (Chapter 2, section V,3).

The anti IgA and anti IgM conjugates detected IgA and IgM antibody to gliadin in the samples. No IgM antibody was detected in the samples sent through the anti IgM columns. The IgA antibody levels were unchanged. The levels of antibody were identical to those measured previously.

Although the total anti gliadin IgA antibody levels were identical to those measured before, low levels of pIgA antibody were measured using the anti SC ELISA.

Firstly, the background colour obtained in the negative control wells (containing gliadin but in which no serum sample was incubated) was high (OD_{405}=0.236) after an 8 hour substrate incubation.

Secondly, the dilution at which pIgA antibody to gliadin could be detected using the anti SC ELISA was low compared to the dilution at

which pIgA antibody to gliadin was detected using the radiolabelled SC.

At the end point of the ELISA for pIgA antibody (OD_{405}=0.15 after backgound subtraction) two subjects who had pIgA antibody to gliadin detected at a dilution of 1/1400 and 1/620 in the RIA only had detectable pIgA antibody to gliadin at a dilution of 1/18 and 1/15 respectively.

Therefore the anti SC ELISA appeared to be less sensitive than the RIA in detecting pIgA antibody to gliadin. This was partly due to the high background which may have been due to the anti SC antibody reacting with gliadin.

Measurement Of Polymeric IgA Antibody To Typhoid LPS

Suspecting that the high background colour might have been due to a unique cross reactivity between the anti SC and gliadin, the use of the anti SC ELISA to measure pIgA antibody to typhoid LPS was explored. The IgM depleted serum sample of a volunteer who had mounted a pIgA antibody response to oral typhoid vaccination (320 Units of pIgA antibody per 100ul detected in the RIA; Chapter 6) was examined.

Plates were coated with typhoid LPS (Chapter 2, section V,3). pIgA antibody to typhoid was

measured in the IgM depleted sample using free SC and the anti SC conjugate under the conditions described (Chapter 4, section III,2,b). Total IgA antibody and IgM antibody was measured using the commercial anti IgA and anti IgM conjugates (as described in Chapter 2, section V,3,b).

IgA and IgM antibody to typhoid LPS was detected in similar quantities to that measured before. IgM depletion by the anti IgM columns was confirmed. At the end point of the anti SC ELISA (OD_{405} minus background = 0.15), pIgA antibody to typhoid LPS was detected at a dilution of 1/32 of the IgM depleted serum sample. Using the ^{125}I labelled SC pIgA antibody could be detected at a dilution of 1/320.

Once again the background colour in the control wells from the interaction of the anti SC directly with typhoid LPS was high (OD_{405}=0.314 at 6 hours)

Therefore the anti SC ELISA was not as sensitive as the RIA in detecting pIgA antibody to typhoid LPS.

d) Limitations Of The ELISA

When the anti SC ELISA was employed to detect pIgA antibody to gliadin or typhoid LPS it appeared to be less sensitive than the RIA (Section c). The

dilutions at which pIgA antibody could be detected in the samples from patients with coeliac disease or healthy volunteers who were immunised with Ty21a were much lower than the dilution titres at which pIgA antibody could be detected using the RIA.

Secondly, the background colour obtained in the ELISA due to the interaction between the anti SC and the antigen coated wells was high. These two factors reduced the sensitivity of the ELISA.

The reduced sensitivity may have been due to dissociation of SC from pIgA during the incubation of anti SC or the substrate. The increased background may have been caused by the anti SC antibody cross reacting with the antigen used to coat the plate.

In the next section the experiments performed to determine the reasons for this reduced sensitivity are described.

The hypothesis that the additional washing steps and additional incubation periods required in the addition of the anti SC conjugate and subsequently the substrate resulted in the dissociation of the bonds between SC and pIgA and IgM was examined.

(d) (i) Comparison Of Binding Of Secretory Component To Polymeric IgA In The Enzyme Linked Immuno Adsorbent Assay And Radioimmunoassay

An assay was performed to compare the binding of SC to solid phase pIgA and IgM in the anti SC ELISA with the binding of SC to the pIgA and IgM in the RIA. Radiolabelled SC was used for the purpose. The same quantity of ^{125}I labelled SC was incubated in the wells of the plates used for the RIA and the ELISA and the binding of the labelled SC to solid phase pIgA and IgM after the performance of the ELISA compared to the binding in the RIA to determine if less SC remained bound on completion of the ELISA.

The assay was performed using 3 micro titre plates. Each plate was coated with doubling dilutions of pIgA and IgM. After the usual blocking and washing 20ng of radiolabelled SC was added to each well of plates 1 and 2.

The binding of the immunoglobulin to the plates was confirmed by performing an ELISA using anti IgA and anti IgM commercial alkaline phosphatase conjugates (Chapter 3, section 1) using the third plate.

After 6 hours at room temperature the first plate was washed 12 times with PBS Tween and the

radioactivity bound to wells measured.

The second plate was washed 6 times and the anti SC conjugate added (1/500 dilution in BSA PBS, 100ul per well). After overnight incubation the plate was washed 10 times and 100ul of substrate added per well. After 6 hours at 37°C the OD_{405} was read, the plate washed twice and the radioactivity remaining bound to the well was measured.

There was less radioactivity bound to the pIgA and IgM coated wells after the performance of the ELISA using anti SC than after the performance of the RIA (Figure 4.2).

Therefore dissociation of SC from pIgA and IgM must have occurred.

(ii) Reasons For The Reduced Sensitivity Of The ELISA: Dissociation Of Bonds Between Secretory Component And Polymeric IgA

It was postulated that the additional washing steps performed during the ELISA and the prolonged incubation with the anti SC conjugate and substrate could cause dissociation of the bonds between SC and pIgA. The experiment described below was performed in order to discover the stage at which dissociation of the binding might have occurred.

^{125}I labelled SC was used to enable the dissociation of SC from pIgA to be measured during

1. The incubation with the anti SC conjugate
2. The subsequent washing
3. The incubation with the substrate

A series of wells of a micro titre plate were coated with pIgA (10ug per ml, 100ul per well). The wells were blocked and washed as usual. Then 20ng of ^{125}I labelled SC in 100ul BSA PBS was incubated in the wells at $4^{o}C$ overnight. The plates were then washed 6 times as usual.

To determine the initial binding of SC to pIgA 8 wells were cut out and the radioactivity bound to the wells measured.

To the remaining wells the anti SC conjugate was added (100ul of a 1/500 dilution of the conjugate in BSA PBS).

At hourly intervals the anti SC was removed from 8 wells at a time. The radioactivity removed with the anti SC was measured as well as the radioactivity remaining bound to the wells. This gave an indication of the dissociation of bound SC from pIgA during the period anti SC was incubated in the wells.

Substrate was added to 8 wells which had been incubated with anti SC for 8 hours. After incubating the substrate in the wells for 6 hours the substrate was removed measuring the radioactivity removed with the substrate in order to gauge the dissociation of radiolabelled SC from pIgA into the solution containing the substrate. The radioactivity remaining bound to the pIgA wells was measured.

The results were as follows:

Radioactivity added to each well = 62,000 cpm (20ng SC)

Radioactivity bound to each well after the overnight incubation of ^{125}I labelled SC = 3300 cpm.

Background binding to BSA blocked wells = 35 cpm.

Radioactivity removed at hourly intervals with the anti SC (an indication of the ^{125}I SC which had dissociated from the pIgA).

Time anti SC incubated (Hours)	Radioactivity removed with anti SC (cpm)	Radioactivity remaining bound (cpm) to wells
1	430	2922
2	465	2459
3	510	2824
4	496	2866
5	429	2476
6	530	2358
7	449	2458
8	506	2336

Therefore there was dissociation of ^{125}I SC from pIgA into the diluent containing the anti SC even after just 1 hours incubation. This did not increase with the length of incubation with anti SC.

There was further dissociation of ^{125}I labelled SC from pIgA when the substrate was incubated in the wells for 6 hours. The radioactivity in the substrate solution removed from each well was 1300 cpm leaving only 1048 cpm

left bound to the wells a 50% dissociation of bound SC into the substrate solution (as 2300 cpm were bound before the incubation of substrate).

In total therefore there was a 69% reduction in bound radioactivity after the incubation of the anti SC conjugate and the substrate as the radioactivity bound to each well had decreased for 3300 cpm to 1048 cpm.

Therefore this experiment revealed that there was a loss of bound SC from pIgA into the solutions containing the anti SC conjugate and into the solution containing the substrate. This would explain the reduced sensitivity of the anti SC ELISA.

e) Attempts To Improve The Sensitivity Of The ELISA

(i) The Use Of Glutaraldehyde

Having in the previous experiment demonstrated dissociation of the SC from pIgA during the ELISA an attempt was made to reduce this dissociation.

The use of glutaraldehyde to fix the SC once it had bound to pIgA or IgM and thereby prevent the subsequent dissociation which occurred with the addition of anti SC and substrate was evaluated. Glutaraldehyde was used in view of its ability to cross-link proteins (Avrameas and

Ternynck, 1969).

This experiment was performed using ^{125}I labelled SC and IgM coated wells. The ELISA was performed using radiolabelled and unlabelled SC in parallel. The usual procedure (outlined in section III,2,b) was followed. The effect of adding glutaraldehyde after the SC incubation or after the incubation of anti SC was examined.

The wells were coated with IgM (5ug/ml). After blocking and washing, doubling dilutions of ^{125}I labelled SC starting from 25ng/well were added in series.

In parallel on another IgM coated plate serial doubling dilutions of unlabelled SC from 5ug/ml were made.

The SC was incubated in the wells for 4½ hours at 4°C. After washing 0.25% glutaraldehyde was incubated in one series of wells of the plates which had been incubated with labelled and unlabelled SC respectively (for 45 minutes at room temperature). The glutaraldehyde was removed from the wells, measuring the radioactivity in the solution. After washing, the anti SC conjugate was incubated in the wells overnight at room temperature. After removing the conjugate 0.25% glutaraldehyde (100ul/well) was added to one series of wells in an attempt to fix the anti

SC which had bound to the SC-IgM on the plate. The glutaraldehyde was removed after an incubation period of 45 minutes at room temperature and the radioactivity removed measured. The plates were then washed and substrate was incubated in the wells at 37^{o}C.

The effects of the glutaraldehyde on the ELISA are illustrated in Figure 4.3. The addition of glutaraldehyde had an adverse effect whether it was added before or after the addition of anti SC (Figure 4.3). The addition of glutaraldehyde before the addition of anti SC to the wells appeared to interfere with the interaction of SC with anti SC. There may have been denaturation of the proteins.

On the plate to which ^{125}I labelled SC was added dissociation of bound ^{125}I SC occurred despite the addition of glutaraldehyde. There was dissociation of ^{125}I SC into the glutaraldehyde solution and there was no difference between the ^{125}I labelled SC left bound to the wells at the completion of the ELISA whether glutaraldehyde was used or not suggesting that glutaraldehyde did not prevent dissociation.

Therefore this approach failed.

(ii) Attempts To Reduce The Background

The second problem encountered in the ELISA was the high background (section III,2,c). As the anti SC antibody was a mouse antibody an attempt was made to reduce the background by blocking with:

1. mouse serum
2. mouse IgM (5ug/ml in BSA PBS)
3. mouse IgG (5ug/ml in BSA PBS)

and comparing the background colour obtained with the mouse sera with a blocking solution of 0.5% BSA PBS.

Micro titre plates were coated with typhoid LPS. After washing 150ul of the blocking solution were incubated in the wells for 1 hour at room temperature before performing the usual assay.

The background colour produced after an 8 hour incubation of the substrate was:

0.16 with 0.5% BSA PBS

0.006 with normal mouse serum

0.03 with mouse IgM

0.035 with mouse IgG.

Therefore the use of mouse serum to block the plates successfully reduced the background. The use of mouse serum as a blocking agent was evaluated in an assay for pIgA antibody to

typhoid LPS in volunteers who had received the oral typhoid vaccine Ty21a.

An ELISA for pIgA to typhoid LPS was performed using IgM depleted serum samples of 2 subjects. Both these subjects developed pIgA antibody to typhoid after vaccination which could be measured by radioimmunoassay (Chapter 6).

The ELISA for anti typhoid pIgA antibody was performed in the usual manner (section III,2,b) comparing the use of 0.5% BSA PBS with the use of mouse serum for blocking.

The end point of the ELISA was the titre giving an OD_{405} of 0.15 (after background substraction) after an 8 hour incubation of the substrate.

The background was reduced from an OD_{405} of 0.14 with0.5% BSA PBS to 0.003 with mouse serum. The table illustrates the dilution titre at which pIgA antibody to typhoid could be measured.

SUBJECT 1

ELISA	1.	1/320 with mouse serum blocking
	2.	1/210 with BSA PBS blocking
RIA		1/1280

SUBJECT 2

ELISA 1. 1/42 with mouse serum blocking

2. 1/32 with BSA PBS blocking

RIA 1/150

Therefore although the background was less with mouse serum and the sensitivity was increased the RIA was more sensitive than the ELISA.

f) Discussion On The Anti SC ELISA

Theoretically there were grounds to hope that the use of free unlabelled SC and an enzyme linked anti SC assay system would enhance the sensitivity and reproducibility of the assay. The enzyme linked system avoids the risk of damage to the SC in the process of radioiodination. The reproducibility of the assay should have been improved due to the greater long term stability of the enzyme conjugate compared to the radiolabelled SC which had the inevitable problem of radioactive decay.

It was hoped that the use of the mouse hybridoma derived monoclonal anti SC antibody to detect SC bound to pIgA would provide a better means of detecting antigen specific pIgA antibody.

The anti SC was able to detect free SC bound to wells of micro titre plates as well as SC bound to wells coated with pIgA or IgM. The ELISA was also used to confirm that SC would not bind to monomeric IgA or IgG bound to microtitre plates.

The specificity of the affinity of SC for pIgA and IgM was demonstrated by the ability of specific anti sera to IgA to inhibit the binding of SC to pIgA and the ability of specific anti sera to IgM to inhibit the binding of SC to IgM and not vice versa.

However, even when wells were coated with purified pIgA the optical density produced when SC bound to pIgA was lower than when SC bound to IgM. This reflects the greater affinity of SC for IgM (Chapter 1, section VIII). The reduced affinity of anti SC antibody to SC bound to pIgA might have been due to a masking of epitopes on SC by the SC pIgA combination. This interaction of anti SC with SC or SC complexed to pIgA has been investigated previously by Woodard et al (1984).

These investigators obtained a panel of hybridoma derived antibodies by immunising mice with free SC or secretory IgA. ELISA's were used to assess antibody

binding. In their studies antibodies with primary specificity for secretory IgA bound secretory IgA assembled *in vitro* but not the free SC used to assemble it. Antibodies with primary specificity for free SC did not bind SC once it was bound to dimeric IgA, suggesting that the free SC epitopes were masked.

In their assays (ELISA peroxidase reactions) the maximum binding of monoclonal antibody to wells containing secretory IgA assembled in vitro (by the interaction of SC with pIgA bound to wells) was only about 10% of the binding to wells coated with native secretory IgA. They suggested that the low saturation level of anti secretory IgA monoclonal antibodies to secretory IgA assembled in vitro reflected the low proportion of pIgA antibody that bound SC in a form detectable with the secretory IgA specific antibody.

The studies described in this chapter conform with these findings.

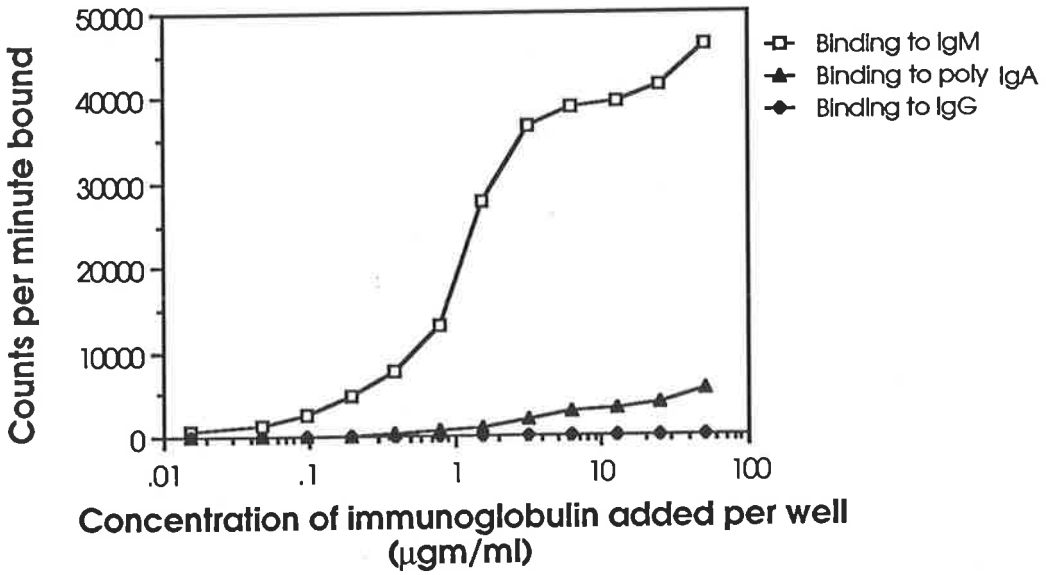
In addition loss of bound SC from both pIgA and IgM during the performance of the additional steps required in the ELISA was demonstrated. Attempts to reduce this loss of bound SC by fixing the SC bound to solid phase pIgA using glutaraldehyde were unsuccessful.

An additional problem was the high background obtained when attempts were made to measure pIgA antibody to typhoid LPS and gliadin using the ELISA. The use of mouse serum reduced the background.

However, when the RIA using ^{125}I labelled SC was compared with the anti SC ELISA, antigen specific pIgA could be detected at a greater dilution of the serum sample using radiolabelled SC.

Therefore, the studies described in subsequent chapters were performed using ^{125}I labelled SC. The ELISA was successfully used to assess the effect of radioiodination on the SC and to provide an alternative method of confirming the specificity of the binding of SC to pIgA.

BINDING OF RADIOLABELLED SECRETORY COMPONENT PREPARED BY THE UNDERDOWN METHOD TO IMMUNOGLOBULINS

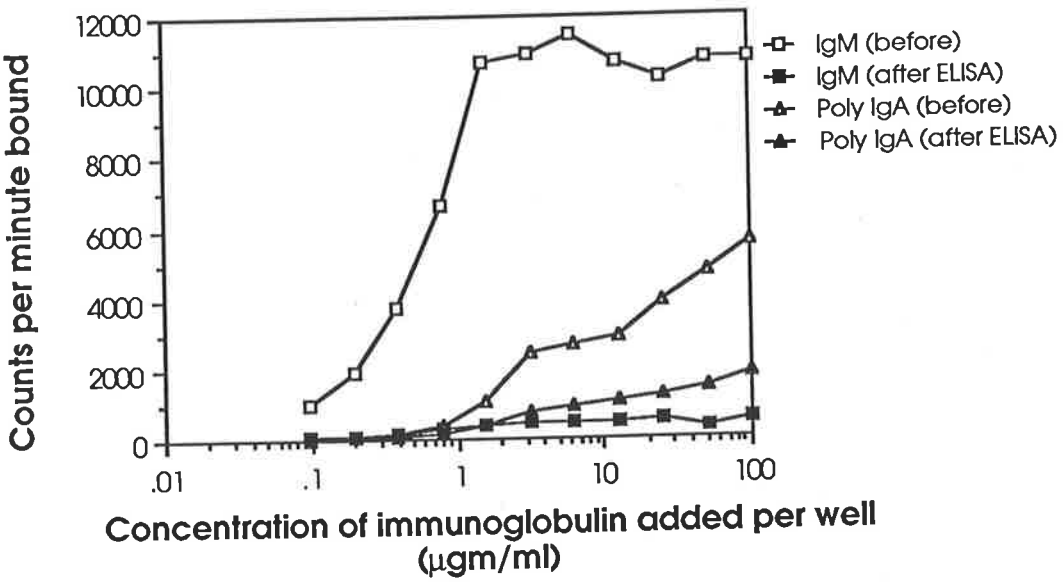


10ng SC per well = 77,250 cpm

Figure 4.1

The binding of 10ng ^{125}I labelled SC (prepared by using IgM sepharose affinity chromatography) to solid phase immunoglobulins. Plates were coated with different concentrations of each immunoglobulin as indicated, before blocking and addition of labelled SC.

THE BINDING OF RADIOLABELLED SECRETORY COMPONENT TO POLYMERIC IgA AND IgM IN THE R.I. A. COMPARED TO THE BINDING IN THE ELISA



20ng SC per well = 70,000 cpm

Figure 4.2

The binding of ^{125}I labelled SC (20ng) to wells coated with pIgA or IgM.

(a) Immediately after the incubation of SC in the wells and washing.

(b) On completion of the ELISA using alkaline phosphatase labelled anti SC antibody to detect the binding of ^{125}I labelled SC to pIgA and IgM.

The plate was coated with different concentrations of pIgA and IgM as indicated before blocking and the addition of labelled SC.

EFFECT OF GLUTARALDEHYDE ON THE SENSITIVITY OF THE ELISA, SHOWING EFFECT ON SC BINDING TO IgM

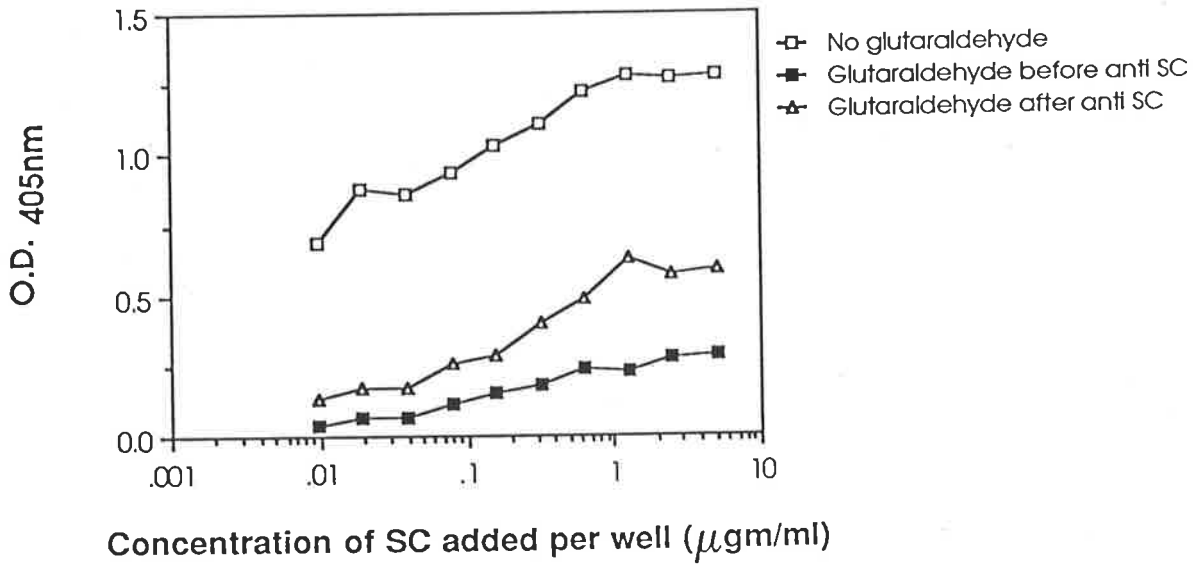


Figure 4.3

The effect of glutaraldehyde on the ability of the anti SC ELISA to detect SC binding to solid phase IgM. Plates were coated by incubating 5ug/ml IgM in the wells. After blocking and washing varying concentrations of SC (as indicated) were incubated in the wells.

The effect of adding 0.25% glutaraldehyde

(a) before the addition of anti SC

(b) after the addition of anti SC

is shown.

CHAPTER 5 - DEVELOPMENT OF THE ASSAY FOR TOTAL POLYMERIC IgA IN SERUM

1. INTRODUCTION

The factors controlling the production and metabolism of pIgA in humans are not clearly understood. Several studies described in this thesis were performed in patients with diseases such as alcoholic liver disease, IgA nephropathy and Crohn's disease in which elevated levels of total serum pIgA had been reported. These reports were discussed in Chapter 1. The quantity of antigen specific pIgA in serum could be influenced by the level of total serum pIgA. In order to make valid comparisons between the level of antigen specific pIgA in patients and normal controls it was essential that an estimate of the total pIgA in the serum samples be made.

With the exception of the SC binding assay described by Newkirk et al (1983) the previous methods used to measure total serum pIgA have involved velocity sedimentation in sucrose density gradients or gel chromatography. These methods were described in Chapter 1.

During the development of the radioimmunoassay for antigen specific pIgA the capacity of pIgA in the liquid phase to inhibit the binding of labelled SC to

wells coated with pIgA or IgM was demonstrated (Chapter 3, section IIIc). This knowledge was utilised to establish a solid phase assay for total serum pIgA using the capacity of pIgA in serum to inhibit the binding of ^{125}I labelled SC to IgM coated wells. This approach was adopted in view of the discovery that soluble pIgA was more effective than IgM in inhibiting the binding of ^{125}I labelled SC to IgM or pIgA coated wells. pIgA could not be purified in sufficient quantity to permit the performance of multiple assays. IgM purified from the serum of patients with Waldenström's macroglobulinaemia could be obtained more readily. Therefore IgM was used to coat the plates.

The assay in its final form is described in Section II. Experiments performed during the development of the assay are described in Section III. Experiments performed to validate the assay are described in Section IV.

II. DESCRIPTION OF THE ASSAY FOR TOTAL POLYMERIC IgA IN SERUM

500ul aliquots of serum were totally depleted of IgM using 2ml anti IgM sepharose affinity columns (see Chapter 3, section II). The total polymeric IgA in the IgM free samples was measured by the ability of

the pIgA to inhibit the binding of 5ng labelled SC to IgM sensitised wells.

Micro titre plates were coated with IgM (0.5ug/ml IgM diluted in 10ug/ml IgG in sodium carbonate coating buffer pH 9.6, adding 100ul per well).

After overnight incubation at $4^{o}C$ the wells were emptied and the unbound sites blocked for 45 minutes with 0.5% BSA PBS, 150ul per well. The plates were then washed.

Serial doubling dilutions of the IgM free serum samples were made in 0.5% BSA PBS. 50ul was added per well. 5ng of ^{125}I labelled SC in 50ul of 0.5% BSA PBS were added to and mixed with the 50ul of diluted serum in each well. After a 24 hour incubation at $4^{o}C$ the plates were washed 6 times with PBS Tween and the bound radioactivity measured.

The uninhibited binding of 5ng labelled SC in 100ul 0.5% BSA PBS to wells sensitised with IgM was measured. In each assay the inhibition by a standard preparation of purified pIgA serially diluted in 0.5% BSA PBS was measured and a standard curve of inhibition obtained (Figure 5.1).

This was used to estimate the pIgA in the samples by extrapolating the dilution of serum producing 50% inhibition of binding to the concentration of purified standard pIgA which produced 50% inhibition of the binding of labelled SC to IgM.

III. Development Of The Assay

The capacity of soluble pIgA to inhibit the binding of labelled SC to IgM coated wells was demonstrated in Chapter 3, section III,1c. In this section the experiments performed to select the most suitable concentration of IgM for coating the plates, the quantity of ^{125}I labelled SC to be added and the optimum conditions for incubating SC are described. In addition, the results of an experiment performed to determine the inter and intra assay variation are presented.

1. Determining The Optimum Concentration Of IgM To Be Used To Coat Wells Of Micro Titre Plates

This experiment was performed to select the concentration of IgM to be used for coating the plates.

Wells were coated by adding 100ul per well of either 5ug/ml, 2ug/ml, 0.5ug/ml or 0.2ug/ml IgM. The IgM was diluted in 10ug/ml IgG diluted in sodium

carbonate buffer pH 9.6. IgG was included in the coating buffer to ensure uniform dispersal of IgM by minimising adsorptive loss to pipettes etc at the low IgM concentration used.

After coating, blocking and washing 50ul of doubling dilutions of purified pIgA were added to each well. Then 10ng of SC in BSA PBS was added and mixed with the pIgA in the wells. After a 4 hour incubation at $4^{o}C$ the wells were emptied, washed and the bound radioactivity measured.

The uninhibited binding to wells coated with 5ug/ml IgM resulted in 24,000cpm of labelled SC binding to the plate (Figure 5.2). 100ug/ml of pIgA in the liquid phase could not completely inhibit the binding of SC. The binding was inhibited to 5000cpm (Figure 5.2).

The uninhibited binding of SC to wells coated with 0.2ug/ml IgM was approximately 1400cpm. At this dilution the standard curve of inhibition by pIgA was erratic. There was unacceptable variation in the degree of uninhibited binding of SC to wells coated with 0.2ug/ml IgM. This suggested that this was too low a concentration of IgM for reliably coating the wells.

Coating wells with 0.5ug/ml IgM to 2ug/ml IgM resulted in a more consistent degree of binding of labelled SC and inhibition of binding with the standard preparation of purified pIgA in the liquid phase (Figure 5.3). The coefficient of variation for estimating the total pIgA in IgM depleted serum sample was 10% within the assay and 11.4% between assays when 0.5ug/ml IgM was used for coating (Section III, 4).

2. Determining The Optimum Concentration Of Labelled SC To Be Added

This experiment was performed to compare the binding of various concentrations of labelled SC to IgM sensitised wells and determine the inhibition of binding induced by pIgA in the liquid phase. This was done in order that the most sensitive assay, capable of detecting minimal quantities of pIgA in serum could be developed.

Wells of a micro titre plate were coated with IgM adding 0.5ug/ml IgM diluted in 10ug/ml IgG in sodium carbonate buffer pH 9.6. After overnight incubation, blocking and washing 50ul of serial dilutions of purified pIgA were added to each well. Then 50ul of labelled SC diluted in BSA PBS was added to and mixed

with the pIgA in the wells.

The addition of 10ng, 5ng or 2.5ng SC to each well was compared. The SC was incubated in the wells for 4 hours at 4°C. The radioactivity bound after washing the plates 6 times was measured.

The addition of less SC per well increased the sensitivity of the assay. The 50% inhibition produced by the same preparation of purified pIgA in this assay was produced by 20ug/ml, 7.4ug/ml and 2.5ug/ml of the polymeric IgA, when 10, 5 or 2.5ng labelled SC was added per well (Figure 5.4).

It was decided to add 5ng SC per well in subsequent assays to avoid the potential adsorptive loss of SC that could occur if 2.5ng of SC was used.

3. Determining The Length Of The Incubation Period For Leaving SC In The Wells

The experiments described so far were performed leaving the labelled SC in the wells for 4 hours. Having shown that the binding of labelled SC to pIgA bound to wells as a solid phase was enhanced if the SC was incubated in the wells for 24 hours at 4°C (Chapter 3, section V,4) it was decided to examine the effect of incubating the SC with the serum in the

wells for 24 hours at 4°C in the inhibition assay.

Wells were coated with 0.5ug/ml IgM. Serial dilutions of pIgA were mixed with 5ng labelled SC and incubated in the wells for 24 hours at 4°C. After 6 washes the radioactivity bound to the wells was measured.

50% of inhibition of binding to IgM was produced by lug/ml purified pIgA in the liquid phase (Figure 5.1). When the SC was incubated in the wells for 4 hours 50% inhibition of binding was produced by 7.4ug/ml pIgA (Section 2, Figure 5.4).

Therefore the longer incubation period enhanced the sensitivity of the assay enabling the detection of a lower concentration of pIgA.

4. Determining The Intra Assay And Inter Assay Variation

The intra and inter assay variation was determined by carrying out measurements on the one sample of 40ug/ml purified pIgA, performing the assay as described in Section II using 5 plates coated with IgM in parallel to determine the intra assay variation and performing the assay using 6 different plates (each on a separate day) to determine the inter assay

variation. The coefficient of variation was 10.16% (n=5) within the assay and 11.4% (n=6) between assays.

IV. VALIDATION OF THE ASSAY FOR TOTAL SERUM POLYMERIC IgA: ASSAY TO CONFIRM THAT IT WAS pIgA IN SERUM THAT INHIBITED THE BINDING OF SC TO IgM

In this experiment samples from 2 subjects who had high titres of pIgA antibody to typhoid LPS after oral vaccination with the Ty21a vaccine were assayed for total polymeric IgA content, before and after passage through an anti-IgA sepharose affinity column. This affinity chromatography was performed to remove IgA class antibody from the sample (the samples had previously been totally depleted of IgM). The inhibition of binding of SC to IgM coated wells by the samples before and after exposure to the anti IgA sepharose was compared.

500ul aliquots of serum of these 2 subjects were first totally depleted of IgM using the anti IgM sepharose columns. As previously described this results in a 10 fold dilution as the unbound protein is eluted in 5ml (see Chapter 3, section II). A 1ml aliquot of this IgM free 5ml sample was then run into a 2ml anti IgA sepharose column. After a 40 min incubation the column was washed with 0.1M phosphate buffer pH 7.6 collecting the first 5ml. This 5ml

sample contained the unbound protein. The 5ml sample was then concentrated back to 1ml using Centricon Amicon filters.

A micro titre plate was coated with 0.5ug/ml IgM, blocked and washed. Then 50ul aliquots of doubling dilutions of the following samples were added to each well.

The samples added:

1. Subject 1. The IgM free sample before exposure to anti IgA sepharose.
2. Subject 1. The IgM free sample after exposure to anti IgA.
3. Subject 2. The IgM free sample before exposure to anti IgA.
4. Subject 2. The IgM free sample after exposure to anti IgA.

50ul serial dilutions of the samples diluted in BSA PBS were added to each well. Then 5ng ^{125}I labelled SC was added to and mixed with the samples. After a 24 hour incubation at $4^{o}C$ the plates were washed 6 times and the radioactivity bound measured. The uninhibited binding of SC to IgM was measured. The inhibition produced by a series of dilutions of purified pIgA was measured to determine the standard

curve of inhibition.

Radial immunodiffusion (Mancini et al, 1965) confirmed the removal of IgM and IgA antibody by the affinity chromatography. The uninhibited binding of SC to IgM left 4400 cpm bound to the wells. lug/ml of the purified pIgA produced 50% inhibition of binding in this assay.

50% of inhibition of binding was produced by a 1/44 dilution of the first subject's IgM depleted serum (Figure 5.5) and a 1/30 dilution of the second subject's IgM depleted serum (Figure 5.5). The total pIgA content of these samples was therefore estimated at 44ug/ml and 20ug/ml respectively.

However after affinity purification with anti IgA sepharose there was no inhibition of binding of SC to IgM. Monomeric IgA does not bind SC (Chapter 3, section III). This experiment therefore confirmed that it was pIgA in the liquid phase in these samples that inhibited the binding of SC to IgM.

IV. SUMMARY

In this chapter the development of a solid phase assay for estimating the total pIgA in human serum has been described. The total serum pIgA in serum was

measured through its capacity to inhibit the binding of radiolabelled SC to IgM coated wells, quantitation being achieved by comparison with the inhibition produced by a preparation of purified polyclonal pIgA. IgM was used to coat the wells as it was easier to prepare in the quantities required for repeated assays than was pIgA itself.

The high affinity of SC for IgM made it difficult to induce complete inhibition of binding of SC to solid phase IgM by the pIgA in serum. A low concentration of IgM was used to coat the plates so that the pIgA could compete favourably for SC. IgG was included in the coating buffer to ensure uniform dispersal of IgM by minimising adsorptive loss to pipettes etc at the low IgM concentration used. As there were minor differences in the binding of labelled SC to different preparations of pIgA and IgM one preparation of purified polyclonal IgA (Chapter 2, section II,3a) was used as the standard in the assays described in subsequent chapters and the same preparation of monoclonal IgM was used to coat the plates.

Immunoadsorbent columns containing anti IgM antibodies were used to rapidly and completely deplete 0.5ml serum samples of IgM yielding samples in which

the SC binding was wholly due to the presence of pIgA. The depletion of IgM was confirmed by ELISA and radial immunodiffusion. Unfortunately this resulted in a 5 fold dilution of the original sample which may prevent the detection of low levels of pIgA.

This assay was successfully used to measure the total serum pIgA in normal subjects and patients with alcoholic liver disease (Chapter 7), coeliac disease, Crohn's disease and IgA nephropathy (Chapter 8).

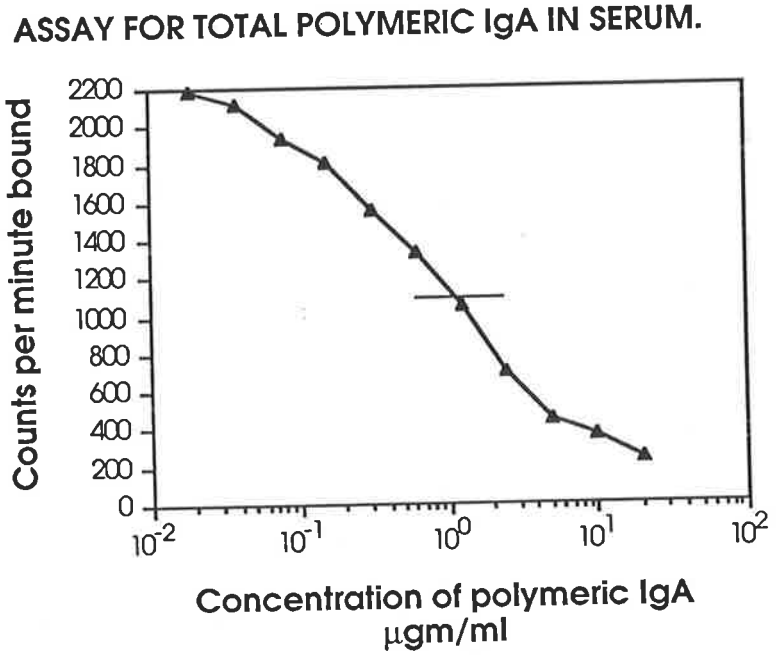


Figure 5.1

Assay for total polymeric IgA in serum. Standard curve depicting inhibition of binding of 5ng ^{125}I labelled SC to solid phase IgM by purified pIgA. 0.5ug/ml IgM was used to coat the plate. The bar indicates 50% binding.

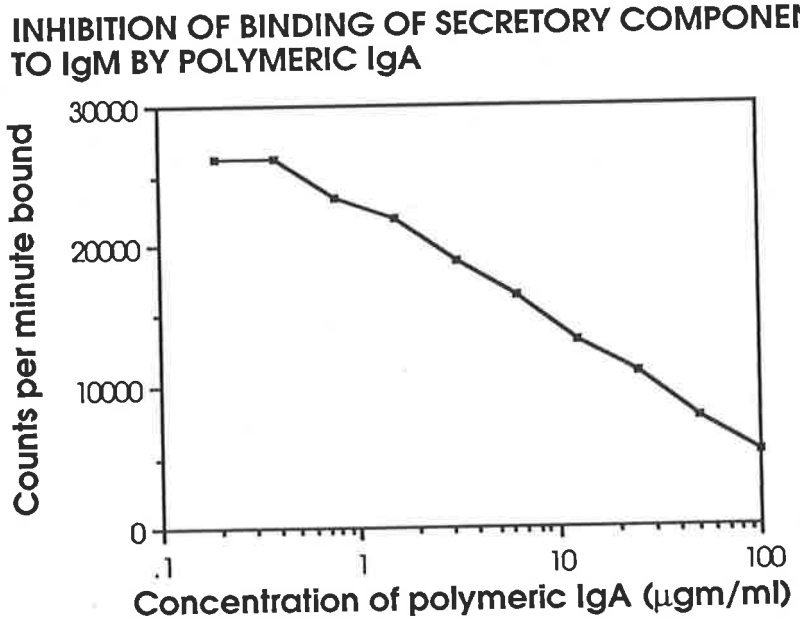


Figure 5.2

The capacity of pIgA to inhibit the binding of 10ng ^{125}I labelled SC to solid phase IgM. 5ug/ml was used to coat the plate. After blocking a range of dilutions of pIgA were added to and mixed with the SC in the IgM coated wells. Uninhibited binding to IgM was 24,000cpm.

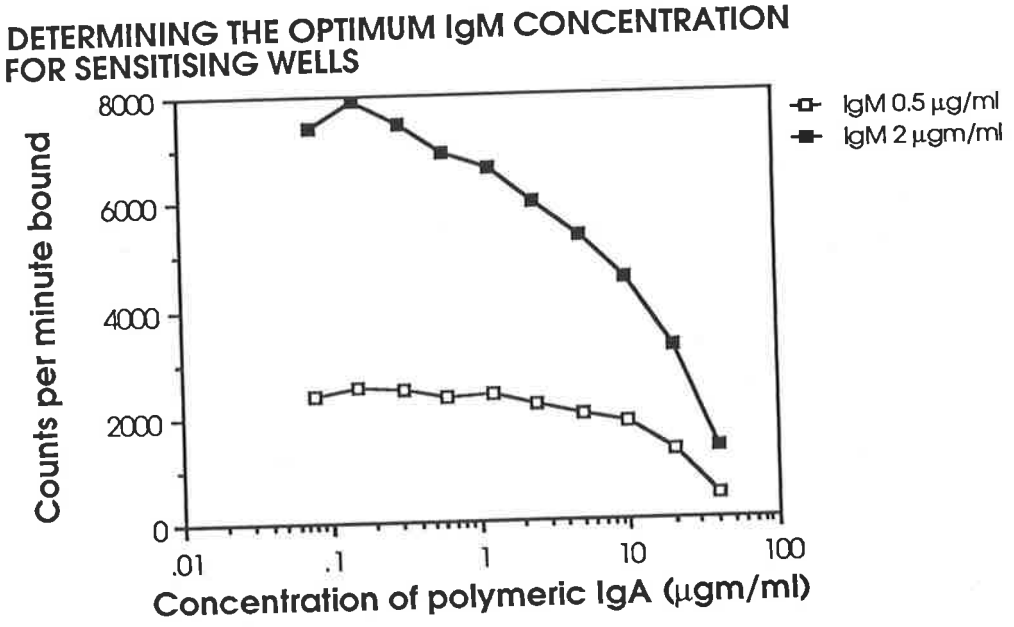


Figure 5.3

The capacity of pIgA to inhibit the binding of 10ng ^{125}I labelled SC to solid phase IgM when either 0.5ug/ml or 2ug/ml was used to coat the plate. After blocking, a range of dilutions of pIgA starting from 40ug/ml were added to and mixed with the labelled SC in the IgM coated wells. Uninhibited binding to 2ug/ml IgM was 8000cpm and to 0.5ug/ml IgM was 2500cpm.

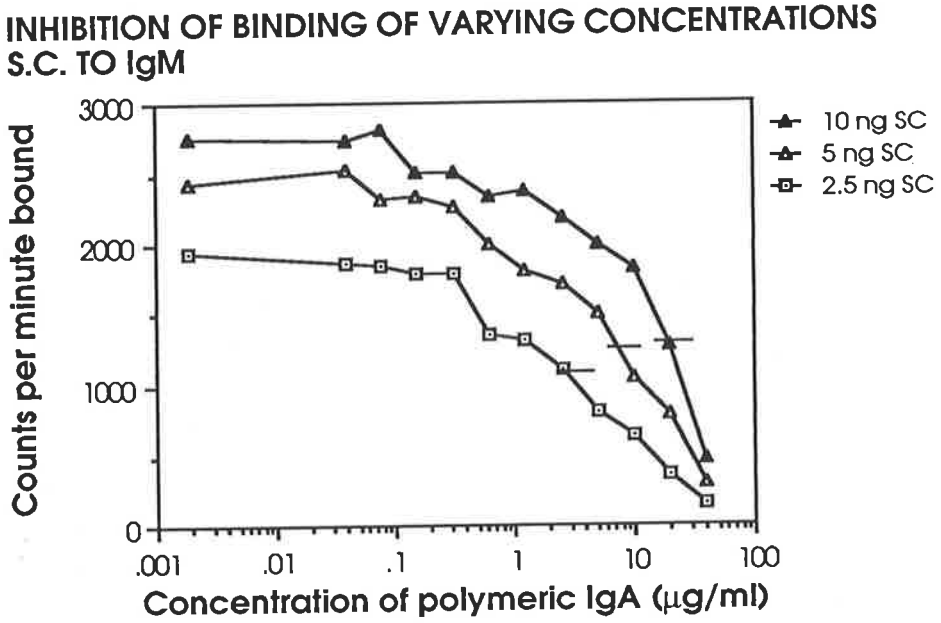


Figure 5.4

The capacity of pIgA to inhibit the binding of varying concentrations of ^{125}I labelled SC to solid phase IgM. The plate was coated with 0.5ug/ml IgM. The binding of 10,5 and 2.5ng SC is shown. The bars indicate 50% binding.

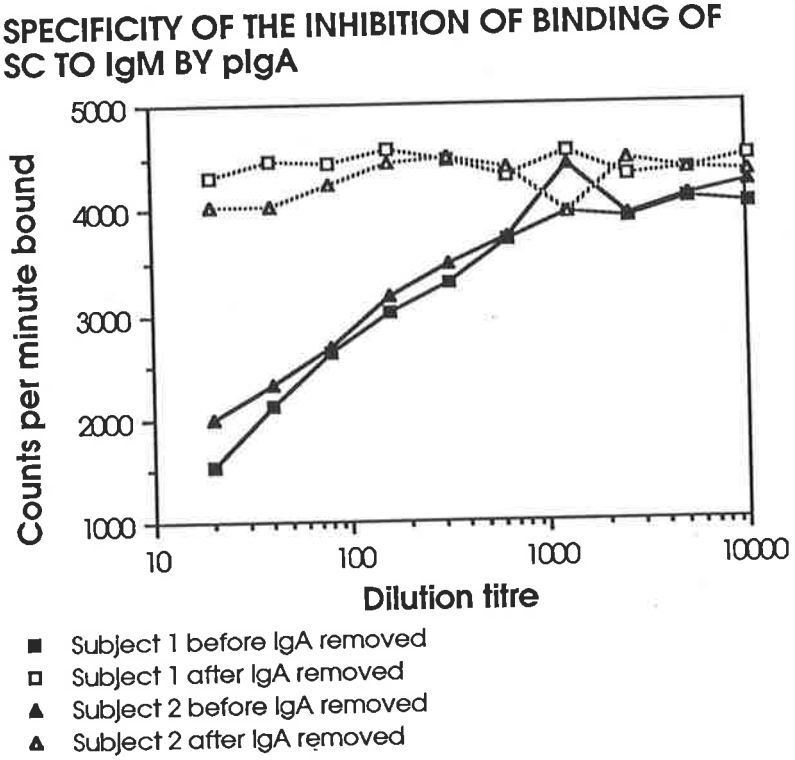


Figure 5.5

The inhibition of binding of 5ng ^{125}I labelled SC to solid phase IgM by 2 serum samples depleted of IgM both before and after IgA depletion using anti-IgA sepharose columns. There was no inhibition after IgA removal.

CHAPTER 6 - A STUDY OF THE SECRETORY IMMUNE SYSTEM IN NORMAL SUBJECTS: THE IMMUNE RESPONE TO TYPHOID VACCINATION

I. INTRODUCTION

The relationship between serum pIgA antibody, total serum IgA antibody (monomeric and pIgA) and IgA antibody in intestinal secretions in humans, was examined by studying the immune response of normal volunteers to typhoid vaccination.

The vaccine used was the live attenuated oral typhoid vaccine Ty21a. This organism a gal E mutant of Salmonella typhi was developed by Germanier and Fürer (1975) using nitrosoguanidine to mutagenise virulent Salmonella typhi strain Ty2 and render it avirulent. The mutant strain lacks the enzyme uridine 5' diphosphate (UDP) galactose 4 epimerase. When galactose is supplied exogenously (as occurs in vivo) the organism accumulates galactose 1 phosphate and uridine diphosphate galactose which cause lysis, rendering it avirulent (Germanier and Furer, 1975).

In preliminary studies in volunteers, Ty21a caused no adverse effects, was genetically stable and protected against experimental infection (Gilman et al, 1977). Five to eight doses of vaccine containing $3\text{-}10 \times 10^{10}$ live organism were ingested without adverse

effects and gave a protection rate of 87% against a challenge dose that caused typhoid fever in 53% of unimmunized controls. A controlled field trial of the vaccine was carried out in Egypt by Wahdan et al (1980, 1982). Three doses of Ty21a vaccine ($1-3x10^9$ viable organism per dose) given after a 1g sodium bicarbonate tablet provided 96% efficacy over 3 years of surveillance. As both the challenge studies (Gilman et al, 1977) and field trial (Wahdan et al, 1982) showed that the vaccine was safe and effective in immunizing against typhoid it was felt that it could offer an important model to study the immune response of humans to the delivery of an antigenic stimulus to the intestine.

When the studies described in this chapter were initiated scientists with whom we collaborated in the Department of Microbiology and Immunology of the University of Adelaide were attempting to produce an oral vaccine against cholera by introducing the DNA coding for the cell surface structures of vibrio cholera into Ty21a using DNA hybridisation technology. It was intended to use the study of the immune response to Ty21a as a model for assessing the immune response to these potential oral vaccines produced by hybridisation. If the measurement of serum pIgA antibody to typhoid proved to be a correlate of the

intestinal immune response it would have facilitated the assessment of these vaccines by demonstrating their capacity to generate an immune response in the intestine without the need for intestinal intubation.

Little information was available on the immune response generated by Ty21a when the studies presented in the chapter commenced. Although one study had shown that serum antibody to typhoid developed in 30% of volunteers after oral vaccination with Ty21a (Black et al, 1983) there was no information regarding the intestinal immune response.

The aim of the initial study (Section II) was to determine if the vaccine generated an immune response in intestinal fluid when administered in a similar dose to that used in previous trials. The effect of varying doses of organisms on the immune response was examined.

Having shown that an intestinal antibody response could be detected after oral vaccination further studies were performed to assess the relationship between IgA antibody in intestinal fluid and serum following oral vaccination.

The results obtained when volunteers were given hybrid cholera-typhoid vaccines are presented in Section III.

Finally a study was performed to examine the relationship between serum pIgA antibody and intestinal IgA antibody following oral and parenteral typhoid vaccination (Section IV).

II. INITIAL STUDY: THE MEASUREMENT OF ANTI-TYPHOID INTESTINAL AND SERUM ANTIBODY FOLLOWING ORAL VACCINATION WITH THE Ty21A VACCINE

1. Introduction

This study examined the immune response generated in intestinal fluid and serum by the oral typhoid vaccine Ty21a. Total intestinal and serum anti-typhoid antibody was measured using anti light chain antibody coupled to alkaline phosphatase.

The aims of this study were: (1) to determine if the oral vaccine generated an intestinal antibody response and (2) to determine the dose that consistently generated intestinal antibody.

2. Materials And Methods

a) Subjects

The subjects were healthy volunteers from whom

informed consent was obtained. They were asked to report any adverse symptoms such as nausea, vomiting, abdominal discomfort or fever. Stool samples were obtained on the third day after vaccination.

b) Vaccine

The vaccine was administered in 2 forms. Seven volunteers received gelatin capsules (Chapter 2, section V,2,a,(i)). A further twenty one volunteers received varying doses of the vaccine in a liquid form prepared in Adelaide (Chapter 2, section V,2,a,(ii)).

c) Vaccination Procedure

Live vaccine is sensitive to gastric acid. Sodium bicarbonate was used to neutralise gastric acid before subjects ingested the vaccine. The 7 volunteers given capsules received 0.8 gm of $NaHCO_3$ in 2 additional capsules 5 minutes before having a dose of the vaccine. The 18 volunteers given the liquid formulation received the vaccine in 50 ml of milk ten minutes after swallowing 1 g of $NaHCO_3$ dissolved in water.

d) Vaccination schedule

All the volunteers received 3 doses of the vaccine on alternate days. The capsules were stored under the conditions stipulated by Professor Germanier

and should have each contained more than 10^9 organisms. The dose given in the liquid form of the vaccine varied; 7 volunteers recieved 3 doses of 10^9 organisms; 5 received 3 doses of 10^{10} organisms and 6 received 3 doses of 10^{11} organisms.

e) Samples

Intestinal fluid and serum specimens were collected before vaccination and 3 weeks post vaccination. Three of the subjects given capsules were studied one year after vaccination.

The collection and storage of samples was performed as described in Chapter 2, section V.1.

f) Assays

Antibody was measured in intestinal fluid and serum using a solid-phase immuno-absorbent assay very similar to an assay which has been characterised and used before (Tsang et al, 1981; LaBrooy et al, 1982) but modified in that an enzyme-linked immunoglobulin probe was used instead of a radio-labelled probe (Bartholomeusz et al, 1986).

The typhoid lipopolysaccharide used to coat the plates was obtained from heat killed Salmonella typhi (Chapter 2, section V, 3,a,(i). The assay was

performed as described in Chapter 2, section V,3,b).

A goat anti human light chain conjugate was used to detect antibody in all immunoglobulin classes.

Each assay included a positive control (the serum of a convalescent typhoid patient with a high antibody titre) and a negative control (the serum of a person never exposed to typhoid). While there was negligible intra-assay variation, the titres varied over a two-fold range in between assays. Because of this inter-assay variation, all samples from each individual were run in the same assay to allow comparison between antibody-titres to be made.

As 100ul volumes of the dilutions of samples were added to each well, the antibody result was expressed as antibody units per 100ul. One unit of antibody was defined as that producing an OD_{405nm} of 0.15. The immunoglobulin content of intestinal fluid is variable therefore the antibody results in intestinal fluid are expressed in terms of units of antibody to typhoid per mgm of immunoglobulin in the samples as determined by single radial immunodiffusion (LaBrooy et al, 1980).

3. Results

The seven volunteers who had a course of the

gelatin coated capsules all showed a clear intestinal antibody response when sampled three weeks after the course of vaccine; mean units per mgm immunoglobulin ± S.E.M. pre vaccine 111.35 ± 54.064; post vaccine 1528.53 ± 257.805; $p < .001$ on paired t test (Figure 6.1). The rise in serum was of a lower order of magnitude with considerable overlap between the levels of antibody measured before and after the vaccine; mean Units per 100ul ± S.E.M. pre vaccine 1625.71 ± 583.698; post vaccine 2677.14 ± 633.019; $p < .01$ on paired t test (Figure 6.2).

In three volunteers who consented to follow-up sampling after 1 year, the intestinal antibody response compared to pre-vaccination samples was still very obvious (Figure 6.3).

The study of the immune response to different doses of the vaccine showed that there was a progressive increase in the intestinal antibody generated by increasing the dose of the vaccine through the range 10^9 - 10^{11} (Figure 6.4).

4. Discussion

This study showed that the oral typhoid vaccine Ty21a generated an immune response at the level of the intestine. An intestinal antibody response that was

consistent and remained detectable a year after vaccination could be generated. The serum antibody response was variable and there was a dissociation between the intestinal and serum antibody response in the samples obtained 3 weeks after vaccination.

The studies with various doses of Ty21a suggested that a dose of around 10^9 organism was needed to generate an immune response but some individuals did not respond to this dose. Increasing the dose to 10^{11} organisms generated an intestinal response in all recipients. There was an increase in both the magnitude and consistency of the response without side effects with the higher dose of 10^{11} organisms.

Having demonstrated that this live oral vaccine could be safely used to generate an intestinal antibody response further studies were performed focussing on the IgA antibody response in serum and intestinal secretions in order to examine the relationship between IgA in the vascular and mucosal compartments.

III. SUMMARY OF THE DATA OBTAINED REGARDING THE SERUM POLYMERIC IgA RESPONSE TO ORAL IMMUNISATION IN VOLUNTEERS GIVEN CANDIDATE HYBRID ORAL CHOLERA TYPHOID VACCINES

The capacity of several candidate oral hybrid cholera typhoid vaccines (Chapter 2, section V,2c) developed by molecular biologists in the microbiology department to generate an immune response to typhoid LPS were evaluated.

A brief summary of some of the information obtained from these studies regarding pIgA antibody is presented. The pIgA antibody response was assessed in 25 volunteers. Six received cholera typhoid vaccine clone V487, 11 received clone EX210 and 8 clone EX363. The vaccination schedules and administration of the oral vaccines were carried out as described in Chapter 6, section II. Samples of serum and intestinal fluid were obtained (as described in Chapter 2, section V,1) before vaccination and 3 weeks after vaccination from the 17 volunteers given V487 and EX210. The ELISA (Chapter 2, section V,3(b) was used to detect anti-typhoid LPS IgA antibody in intestinal fluid and serum. The SC binding assay (Chapter 3, section II) was used to detect anti-typhoid LPS pIgA antibody in serum.

No pIgA antibody to typhoid LPS could be detected in the sera obtained before vaccination. pIgA antibody to typhoid LPS could be detected in 12 of these 17 volunteers after vaccination (35.8 Units per 100ul ± 56) (Figure 6.5).

An anti-typhoid intestinal IgA antibody response was seen in 15 of the 17 subjects (mean antibody levels ±SD, pre- and post-vaccination were 1317±1316 and 8330±5878 units per mg). This difference was significant (t-4.88, p=0.0002). There was a significant rise in total serum IgA antibody to typhoid after vaccination (82±71 to 537±359 Units per 100ul; t=5.05, p=0.0001). Three of the 17 did not have a serum IgA anti typhoid antibody response.

There was no correlation between the anti-typhoid serum pIgA antibody level and intestinal anti-typhoid IgA antibody after vaccination (rs=0.229 not significant at the .05 level of significance). Neither was there a correlation between the total anti-typhoid serum IgA antibody and the intestinal IgA antibody (rs=0.262). There was a correlation between the anti-typhoid serum pIgA antibody and the anti-typhoid total serum IgA antibody in the sample obtained 3 weeks after vaccination (rs=0.49, significant value at the 0.05 level of significance =

0.425).

Total pIgA was measured in the serum obtained before vaccination and 3 weeks later. There was no difference (mean level total pIgA=55ug/ml ± 36).

Therefore in summary only 12 of the 15 subjects who mounted an intestinal IgA antibody response to typhoid LPS had anti-typhoid serum pIgA antibody 3 weeks after vaccination.

To determine if the pIgA antibody response might have occurred earlier, samples of serum were obtained from 8 volunteers given the EX363 cholera typhoid vaccine clone on the 7th and 14th day after vaccination. Intestinal antibody was not examined.

No anti-typhoid pIgA antibody was detected before vaccination. Anti-typhoid serum pIgA antibody was found in all 8 volunteers on the 7th and 14th days after vaccination (78±62 and 67.5±51 units per 100ul; Figure 6.6). The total serum IgA antibody to typhoid LPS also rose after vaccination in the 8 subjects (serum IgA anti-typhoid antibody pre vaccine 83±80; day 7 post vaccination 373±147; day 14 post vaccination 463±284 units/100ul respectively). This rise in total serum IgA antibody was significant

(t=4.83, p=0.0018).

This suggested that the serum pIgA antibody response to oral vaccines was an early and perhaps short lived response. Therefore a series of studies to investigate the kinetics of the pIgA response were performed. These studies are described in the next section.

IV. THE SERUM POLYMERIC IgA ANTIBODY RESPONSE TO TYPHOID VACCINATION; IT'S RELATIONSHIP TO THE INTESTINAL IgA RESPONSE

1. Introduction

In this study the magnitude and kinetics of the serum pIgA antibody response to typhoid vaccination was examined. The IgA response in intestinal fluid and the total serum IgA response (monomeric and pIgA) to this antigenic stimulus was also studied. The effect of oral vaccination with Ty21a was compared to that of parenteral immunisation using the conventional heat killed typhoid vaccine. The central aim was to determine if there was a relationship between the generation of an anti-typhoid serum pIgA antibody response and the generation of intestinal anti-typhoid antibody.

2. Materials And Methods

a) Subjects

Twenty healthy volunteers (mean age 25 years; range 19 to 52) took part in these studies. None of them had a history of typhoid fever and none had previously been immunised against the disease. They gave written informed consent for the study.

b) Vaccine

The oral Ty21a vaccine was supplied to us in the form of lyophilised oral doses each comprising 10^{11} viable organisms (Chapter 2, section V,2,a,(iii)). Volunteers reported for the vaccine after an 8 hour fast. After drinking 50ml of 2% sodium bicarbonate to neutralise stomach acid, they ingested the vaccine resuspended in 50ml of 0.9% sodium chloride.

The parenteral vaccine used in this study was the one commercially available (Chapter 2, section V,2,b). Each dose consisted of 5×10^8 smooth *Salmonella typhi* organisms that had been heat-killed and was administered in 0.5 ml as a sub-cutaneous injection.

c) Study Design

There were three groups of volunteers. Groups 1 and 2 received oral Ty21a according to an identical

schedule but in group 2 the intestinal antibody response was followed to 6 weeks to better define the kinetics of the immune response which was followed to 3 weeks in group 1.

Group 1. The 7 subjects in Group 1 each received 3 doses of oral vaccine 48 hours apart. Samples of intestinal fluid were obtained before vaccination and 7, 14 and 21 days after the first dose. Samples of blood were obtained every 3 to 4 days after vaccination.

Group 2. The 7 subjects in this group also received 3 doses of oral Ty21a 48 hours apart. Samples of intestinal fluid were obtained before vaccination and on days 15, 29 and 43 after vaccination. Blood was obtained on days 0,8,12,15,29 and 43.

Group 3. These 6 subjects were initially vaccinated by subcutaneous injection with 0.5×10^9 of heat killed Salmonella typhi on day 0 and day 14. Three doses of Ty21a were administered orally on day 33, 35 and 38. Each dose contained $1x10^{11}$ organisms.

Intestinal fluid was obtained from these volunteers before vaccination and on days 31 (3 weeks

after the second parenteral vaccination and just before the commencement of oral vaccination) and 52 (3 weeks after the commencement of oral vaccination).

Blood was obtained at regular intervals (3 to 7 days apart) up to day 54 following the initial injections.

d) Samples

Samples of intestinal fluid and serum were obtained and stored as described in Chapter 2, section V,1.

e) Assays

The antigen used was Salmonella typhi Ty2 LPS (Sigma) that had been linked to methylated bovine serum albumin (Chapter 2, section V,3,a,(i)).

The ELISA for IgA antibody in intestinal fluid and serum to S.typhi LPS was performed as described in Chapter 2, section V,3,b.

The RIA for serum pIgA antibody to S.typhi LPS was performed as described in Chapter 3, section II.

The RIA for total serum pIgA was performed as described in Chapter 5, section II.

Immunoglobulin concentrations were measured by single radial immunodiffusion (Chapter 2, section V,4).

3. Results

Group 1. Serum pIgA antibody to typhoid LPS was detected in 4 of the 7 subjects after oral immunisation (Figure 6.7A). The peak level of serum anti-typhoid pIgA antibody after vaccination was 55.5±70.6 units/100ul (mean ±SD). The time course of the serum pIgA response, the intestinal IgA response and the total serum IgA response is illustrated in Figure 6.8. All 7 subjects had an intestinal response to the vaccine (1856±3454 and 8826±6689 units of antibody/mg pre- and peak post-vaccine respectively; Figure 6.7C). This difference was statistically significant (t=4.086, p=.0064). The peak intestinal anti-typhoid IgA antibody was found on day 14 (3 subjects) or day 21 (4 subjects). With the exception of one subject, serum pIgA antibody could not be detected 21 days after vaccination, despite the prevalence of anti-typhoid IgA antibody in intestinal fluid at this time. Although there was a rise in total serum IgA anti-typhoid antibody in all 7 subjects (from 43±26 pre vaccination to 541±535 antibody units/100ul post vaccination; Figure 6.7B),

this difference just failed to reach significance (t=2.434, p=0.0509). The pIgA response closely paralleled the total serum IgA response (Figure 6.8).

Group 2. Anti-typhoid serum pIgA antibody was detected after oral vaccination in 3 of the 7 subjects (27.3 ± 55 units/100ul; Figure 6.9A). All 7 subjects exhibited a significant rise in intestinal IgA antibody to typhoid after vaccination (Figure 6.9C). The mean antibody levels pre-vaccination and post-vaccination were 131 ± 223 and 9867 ± 7315 units per mg, respectively. There was a rise in total serum IgA antibody to typhoid after vaccination (76 ± 81 cf. 323 ± 283 units/100ul; Figure 6.9B). This difference was significant (t=2.588, p=0.0413). The time course of the antibody response is illustrated in Figure 6.10. Anti-typhoid serum pIgA antibody levels peaked between days 12 and 15 post vaccination and could not be detected in serum by day 29 although anti-typhoid IgA antibody was still present in intestinal fluid collected on day 29 and 43 (5345 ± 8701 and 5416 ± 7680 units/mg respectively).

Overall, considering the 14 volunteers vaccinated by the oral route, a significant rise in intestinal IgA antibody and total serum IgA antibody to typhoid was evident after vaccination (Intestinal IgA: 993 ±

2516 units/mg pre; 9349 ± 6754 units/mg, post; t=5.25, p=.0002; total serum IgA: 59.5 ± 60 units/100ul pre, 432 ± 427 units/100ul post; t=3.271, p=.0061).

None of the volunteers had pIgA antibody to typhoid detectable before vaccination. Seven developed pIgA antibody to typhoid after oral vaccination (46.4 ± 59 units/100ul; post vaccination). This difference was significant (t=2.477, p=.0278). There was a significant correlation between the level of anti-typhoid serum pIgA antibody and total serum anti-typhoid IgA antibody found in these 14 volunteers after oral vaccination (rs=0.841, critical value of rs at the .05 level of significance for the one-tailed test = 0.456, Figure 6.11A). On the other hand no correlation was evident between the peak level of intestinal IgA antibody and serum pIgA antibody to typhoid after vaccination (rs=0.227, critical value at .05 level of significance =0.456, Figure 6.11B). Neither was there a correlation between the intestinal IgA antibody levels and the total antityphoid serum IgA antibody after vaccination (rs=0.373 critical value of rs at .05 level of significance =0.456, Figure 6.11C).

Group 3. Three of the 6 volunteers vaccinated by injection had detectable levels of serum pIgA against

S.typhi LPS before vaccination (28.3 ± 36.5 antibody units/100ul). pIgA antibody rose in all 6 volunteers (130.5±94.6 Units/100ul; mean±SD; Figure 6.12A) after the injection (t=2.302;p-0.069 in the paired t test; p<0.05 in the Wilcoxon signed rank test). The peak level of pIgA antibody to typhoid was seen 10 to 13 days after injection in 5 volunteers and on the 10th day after the second injection in the sixth. The level of serum pIgA antibody to typhoid declined by day 33 (47.8 ± 35.4 units/100ul). A further rise in anti-typhoid serum pIgA occurred after the oral vaccine was administered with the peak level seen on day 41 (3 subjects) or day 44 (3 subjects) after the injection (or day 9 and 11 after the first oral dose respectively). The difference between the peak pIgA antibody levels before and after the oral vaccine was administered (47.8 ± 35.4 and 127.2 ± 73.2 units/100ul) was significant (t=2.644, p=0.0457).

There was no significant difference in intestinal IgA antibody to typhoid LPS after the parenteral vaccination (344 ± 415 pre and 1124 ± 975 post; Units per mg, t=2.074, p=0.0927). However, all 6 subjects had a rise in intestinal IgA antibody to typhoid after oral immunisation (Figure 6.12C) from 1124 ± 975 before to 5410 ± 1615 units per mg (mean ± S.D.) after immunisation. This difference was significant

(t=6.734, p=0.0011). The mean total serum IgA antibody levels to typhoid before and after the injection were 166 ± 135 and 858 ± 341 units per 100ul (mean ± S.D.) respectively (Figure 6.12B). This difference was significant (t=4.32; p=0.0076). The level of serum IgA antibody hardly declined before the oral vaccine was administered. The levels of antibody before and after oral vaccination were 808 ± 320 and 777 ± 553 units per 100ul respectively (t=.1696, p=0.8719).

Total Serum pIgA Levels

The mean total serum pIgA concentration in these 20 volunteers was 55ug/ml (range up to 90ug/ml).

4. Discussion

This study suggests that a serum pIgA antibody response can be produced by delivery of an immune stimulus to either the mucosal or vascular compartments in healthy subjects and that the regulation of the production of serum pIgA and intestinal IgA are independent.

A serum pIgA antibody response was generated by delivery of an antigenic stimulus to the intestine. Seven of the 14 volunters given the attenuated live oral typhoid vaccine developed serum pIgA antibody to

typhoid after vaccination. This pIgA response occurred early and was short lived. The peak anti-typhoid serum pIgA level was detected 10 to 14 days after vaccination. A serum pIgA antibody response was also produced by parenteral immunization with killed typhoid organisms. In contrast, an intestinal immune response was only generated by oral vaccination. Intestinal IgA antibody was demonstrated in all the volunteers given 3 doses of 10^{11} organisms; findings consistent with the previous study (Chapter 6, section II). In the earlier study, intestinal IgA antibody to typhoid was detected as long as one year after vaccination. In the present study, anti-typhoid IgA antibody was still present in intestinal fluid 54 days after vaccination at a time when the serum pIgA antibody had disappeared.

There was no significant change in intestinal typhoid specific IgA antibody after parenteral vaccination. This is consistent with previous reports that parenteral immunization with various antigens usually does not stimulate a secretory IgA immune response in external secretions (Heremans, 1974a; Ogra et al, 1968). Intestinal IgA antibody to typhoid was detected in these same subjects after they were given the oral vaccine. Three of the volunteers given the parenteral killed organism had detectable levels of

serum pIgA antibody to typhoid before they received the vaccine and mounted the greatest response to it. These same subjects also had the highest level of anti-typhoid total serum IgA antibody before vaccination. Although they had no recollection of being previously immunised to the organism, these results raise that possibility.

This study confirmed previous observations that even when the IgA class of antibody is examined after intestinal infection or immunisation, there is no correlation between the intestinal antibody response and serum antibody (LaBrooy et al, 1980; Chau et al, 1981; Jertborn, Svennerholm and Holmgren, 1986). In the present study there was no correlation between the peak level of anti-typhoid IgA antibody in intestinal fluid and the anti-typhoid serum pIgA antibody or total anti-typhoid serum IgA antibody. There was a close relationship between the serum pIgA antibody response and the total serum IgA antibody response. The kinetics of the pIgA response parallelled the serum IgA antibody response after oral immunisation. These results also confirm and extend the observations of Mascart-Lemone and colleagues (1987) who demonstrated the occurrence of a short-lived serum polymeric IgA response that paralleled the monomeric IgA response in serum following parenteral vaccination

with Tetanus toxoid. They suggested that the pIgA was produced by lymphocytes in the regional lymph nodes draining the site of vaccination (Mascart-Lemone et al, 1987). They did not address the question of the relationship of these serum responses to that seen in the intestine. On the other hand Brown et al (1985) reported that experimental secondary infection with Influenza A virus, a virus that infects mucosal surfaces resulted in a predominantly pIgA antibody response to the viral haemagglutinin in serum and suggested that the pIgA antibody was derived from secretory surfaces.

Although the present study suggests strongly that the pIgA antibody detected in the circulation after vaccination, whether oral or parenteral, is not derived from the intestinal mucosa, it does not shed light directly on where it comes from. Kutteh et al (1982a) have demonstrated that up to 40% of the IgA secreted by activated lymph-node cells is polymeric. Lymphocytes in peripheral blood produce pIgA both spontaneously and after mitogen stimulation (Kutteh et al, 1980) though these cells may be migrating through the blood stream prior to homing back to the mucosa having been initially activated by antigen in a mucosal site (McDermott and Bienenstock, 1979; Czerkinsky et al, 1987; Forrest, 1988). The appearance

of antigen-specific pIgA in the circulation and not in intestinal secretions after parenteral vaccination also argues in favour of serum pIgA originating in extra-mucosal sites.

These findings meant that the detection or measurement of serum pIgA antibody could not be used as a reliable correlate of the intestinal IgA antibody response in healthy subjects. Further studies were performed in disease states and are described in subsequent chapters.

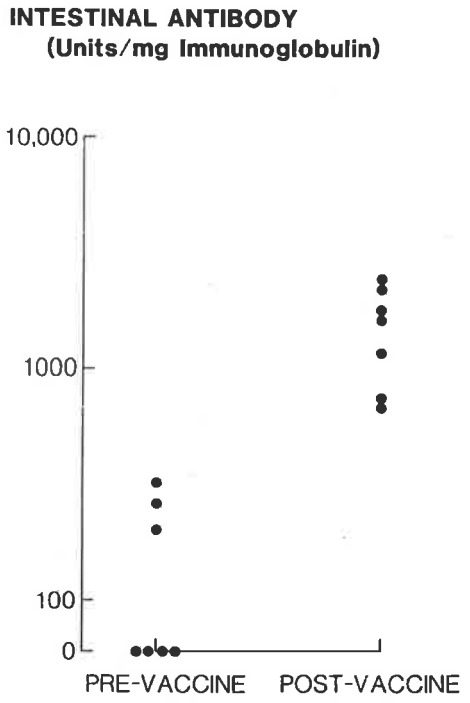


Figure 6.1

Intestinal antibody to S.typhi LPS before and three weeks after vaccination in seven volunteers given gelatin coated capsules of Ty21a.

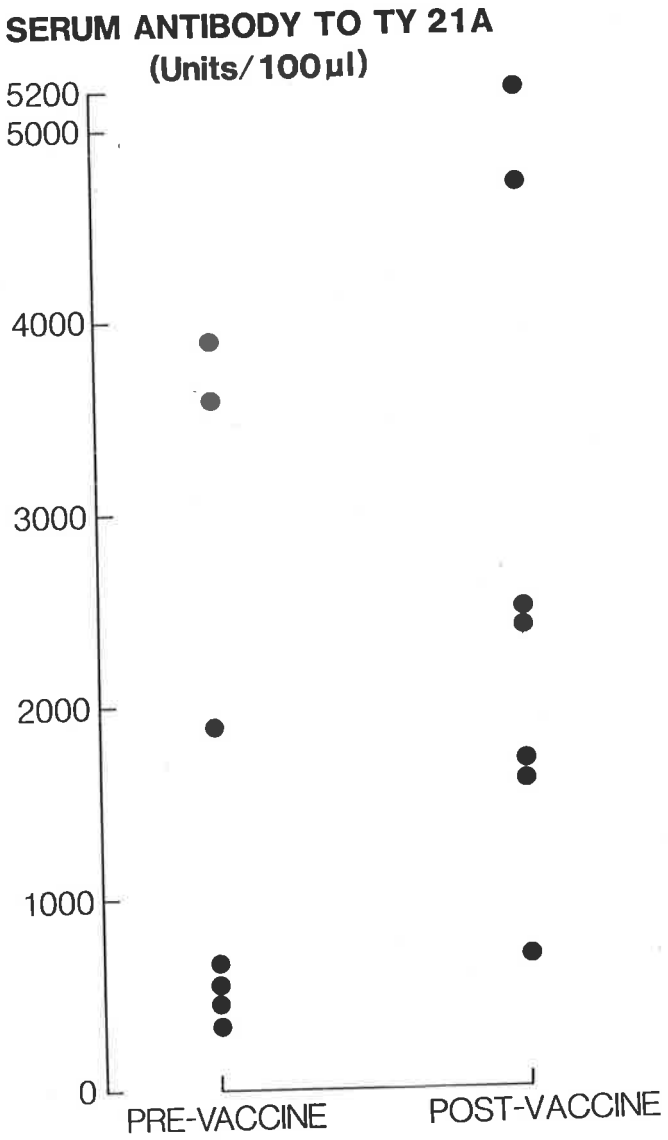


Figure 6.2

Serum antibody to S.typhi LPS before and three weeks after vaccination in seven volunteers given gelatin coated capsules of Ty21a.

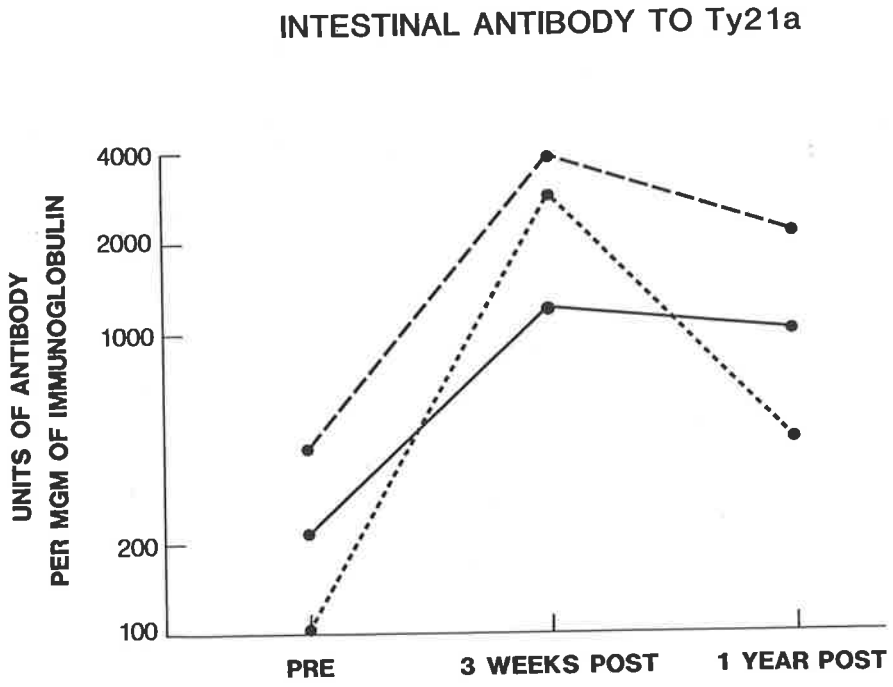


Figure 6.3

Intestinal antibody pre vaccination

(a) three weeks post-vaccination and

(b) one year post-vaccination in three volunteers given gelatin coated capsules of Ty21a.

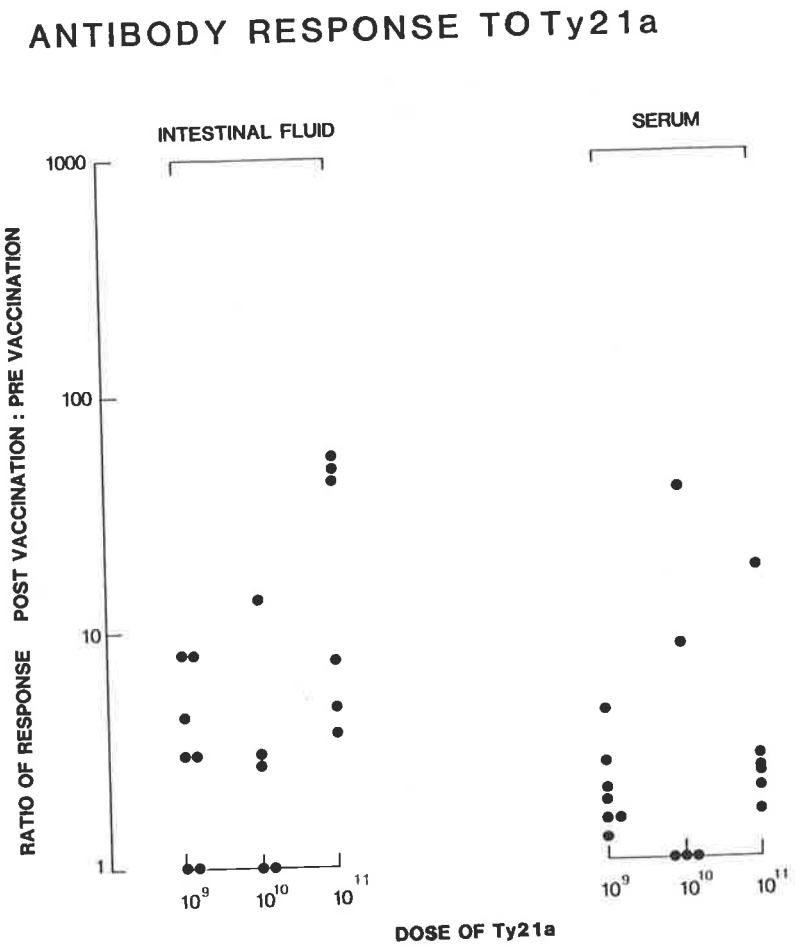


Figure 6.4

The antibody response in (a) intestinal fluid and (b) serum expressed as the ratio of the antibody levels post-vaccination to pre-vaccination in the volunteers given varying doses of the liquid formulation of the vaccine.

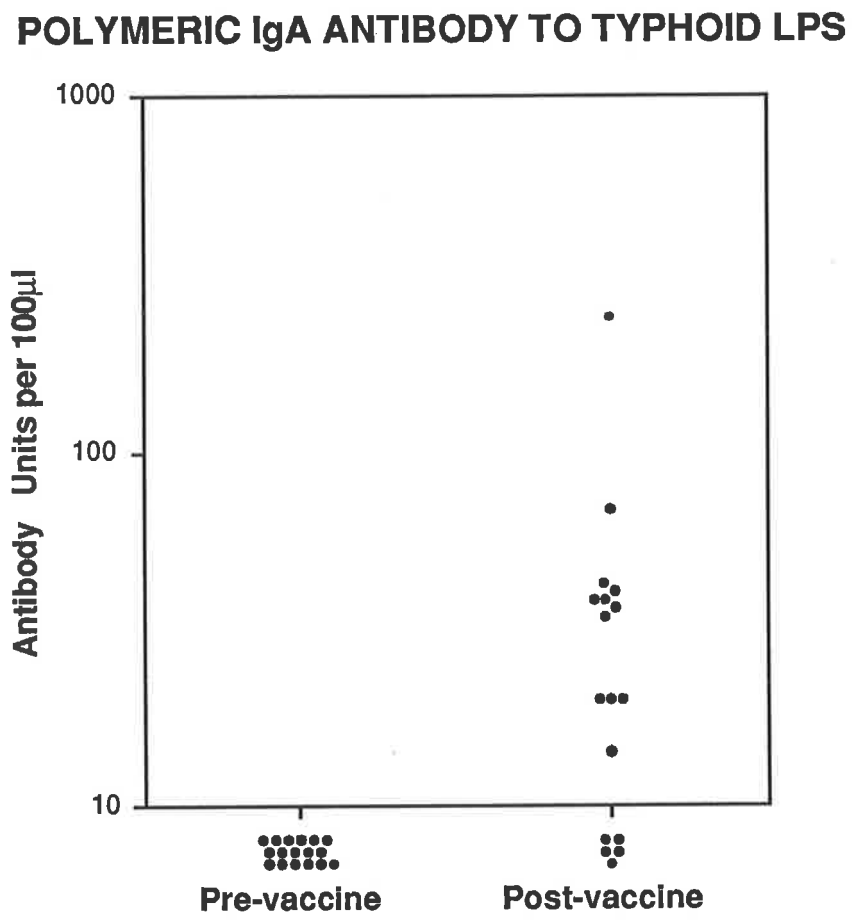


Figure 6.5

Typhoid LPS-specific pIgA antibody pre-vaccination and three weeks post-vaccination in seventeen volunteers given hybrid cholera-typhoid oral vaccines.

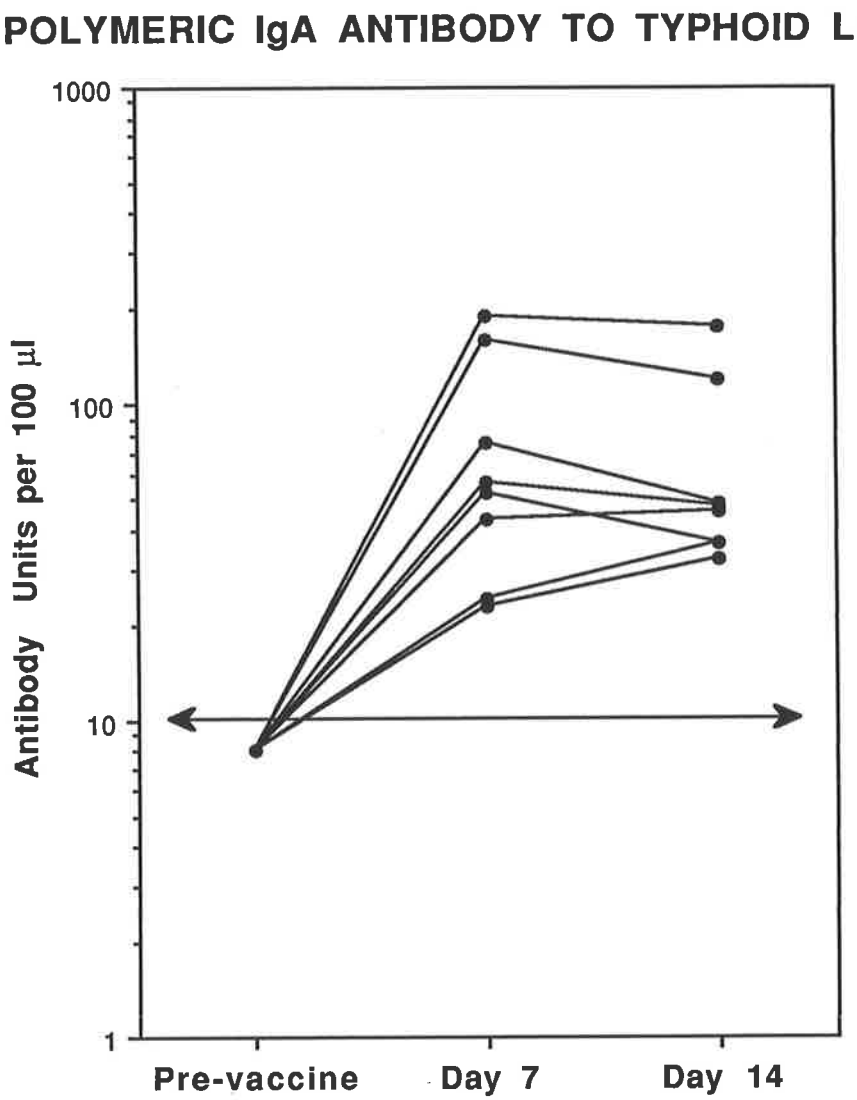


Figure 6.6

Typhoid LPS-specific pIgA antibody in eight volunteers given the hybrid cholera typhoid vaccine clone EX363, (a) pre-vaccination, (b) 7 days post post-vaccination and (c) 14 days post-vaccination.

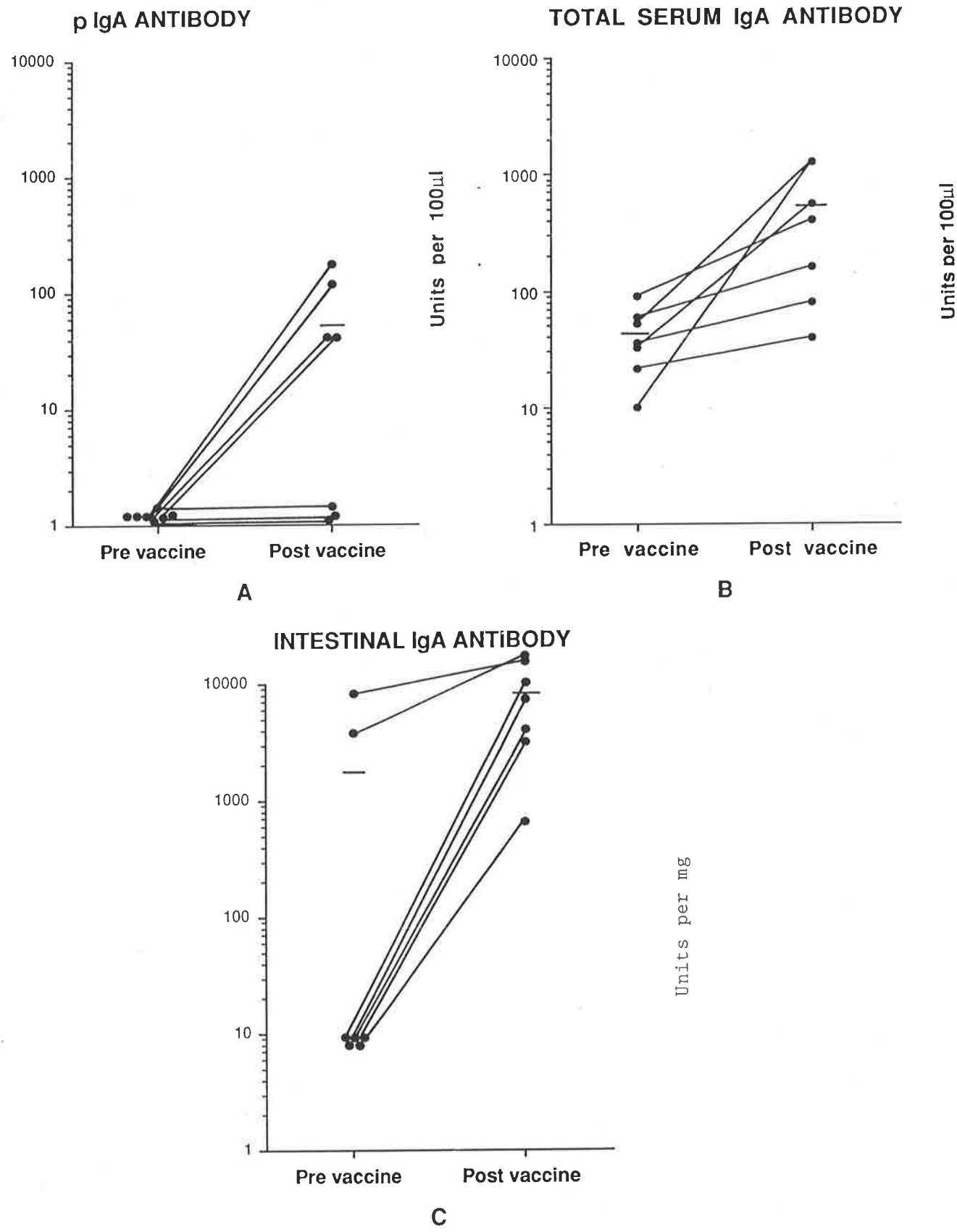


Figure 6.7

Typhoid LPS-specific IgA antibody in seven volunteers (Group 1) given the oral typhoid vaccine Ty21a.

(a) pIgA antibody

(b) Total serum IgA antibody

(c) Intestinal IgA antibody

The peak levels of antibody post-vaccination are shown. Horizontal bars indicate means.

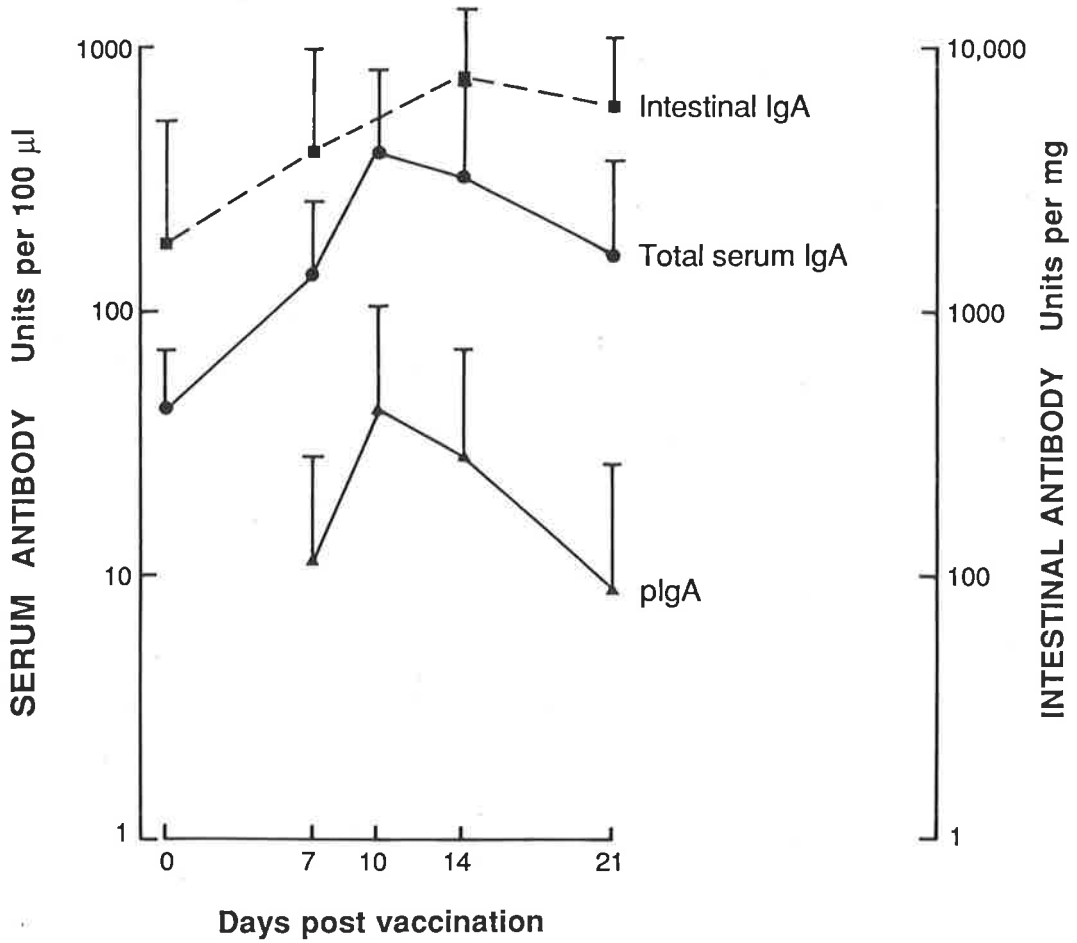


Figure 6.8

The time course of the typhoid LPS-specific IgA response to oral typhoid vaccination up to 21 days post-vaccination in seven subjects (Group 1). Vertical bars represent 1 S.D.

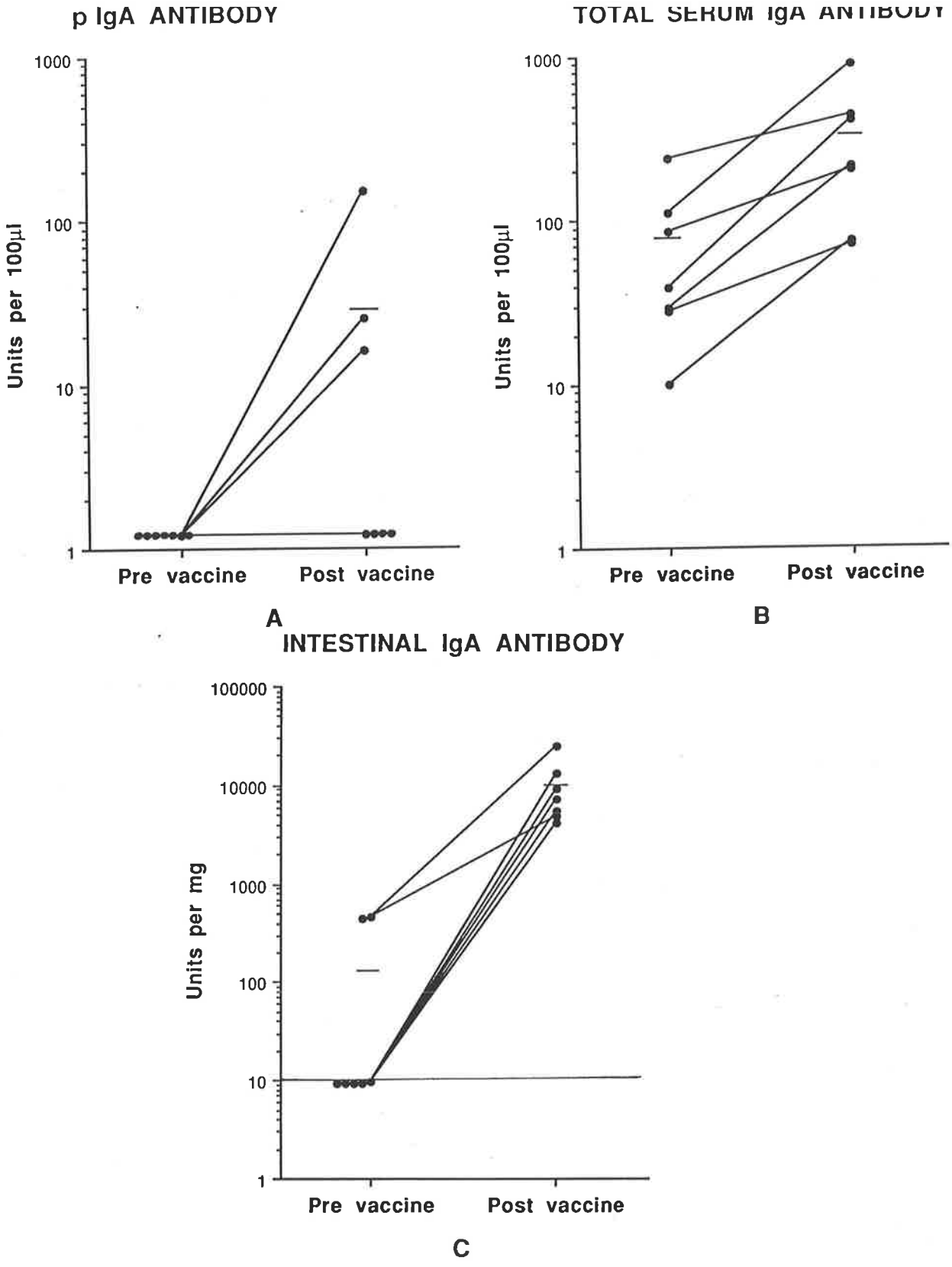


Figure 6.9

Typhoid LPS-specific IgA antibody in seven volunteers (Group 2) given the oral typhoid vaccine Ty21A.

(a) pIgA antibody

(b) Total serum IgA antibody

(c) Intestinal IgA antibody

The peak levels of antibody post-vaccination are shown. Horizontal bars indicate means.

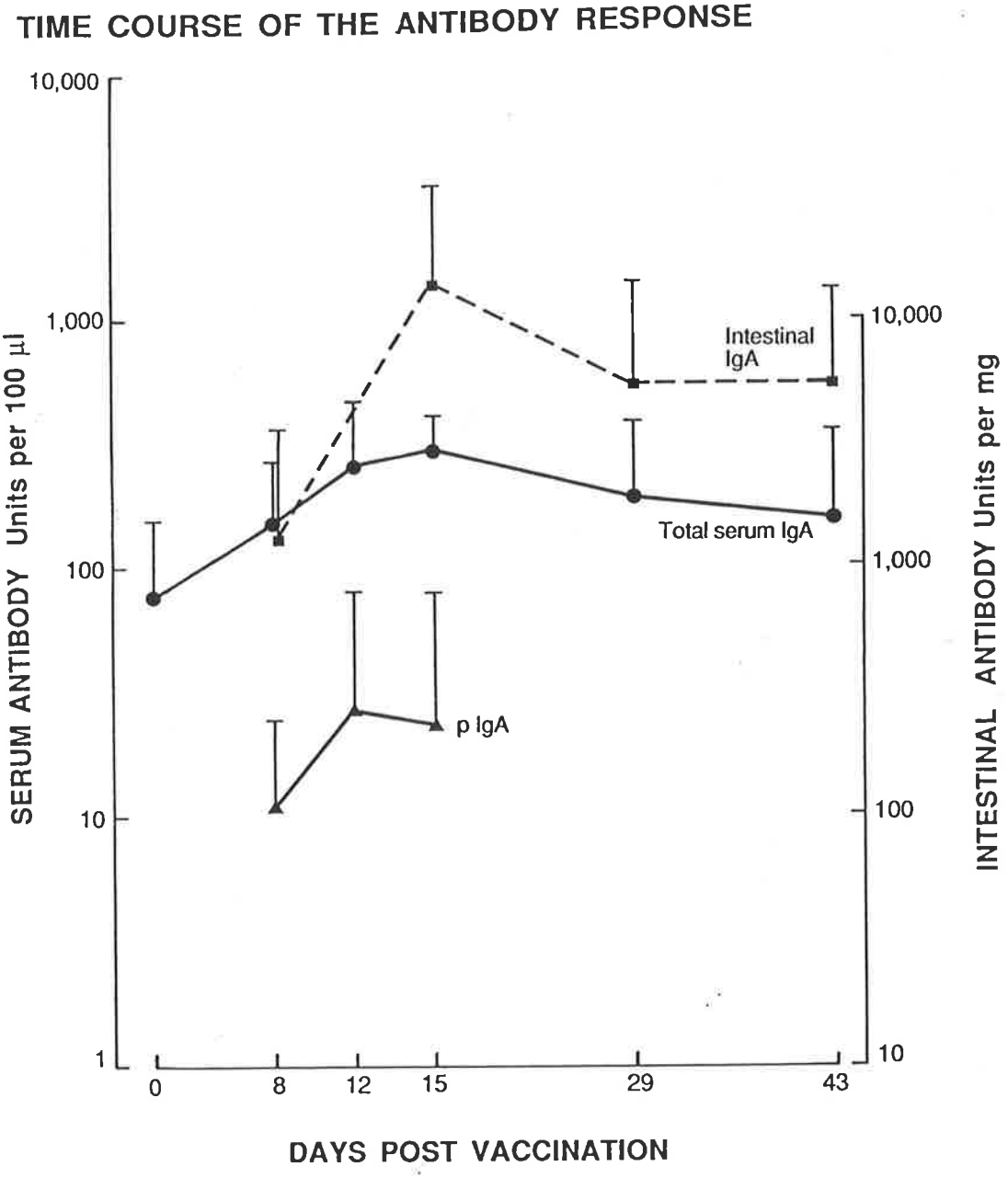


Figure 6.10

The time course of the typhoid LPS-specific IgA response to oral typhoid vaccination in seven subjects (Group 2) where the response was followed up to 43 days post-vaccination. Vertical bars indicate 1 S.D.

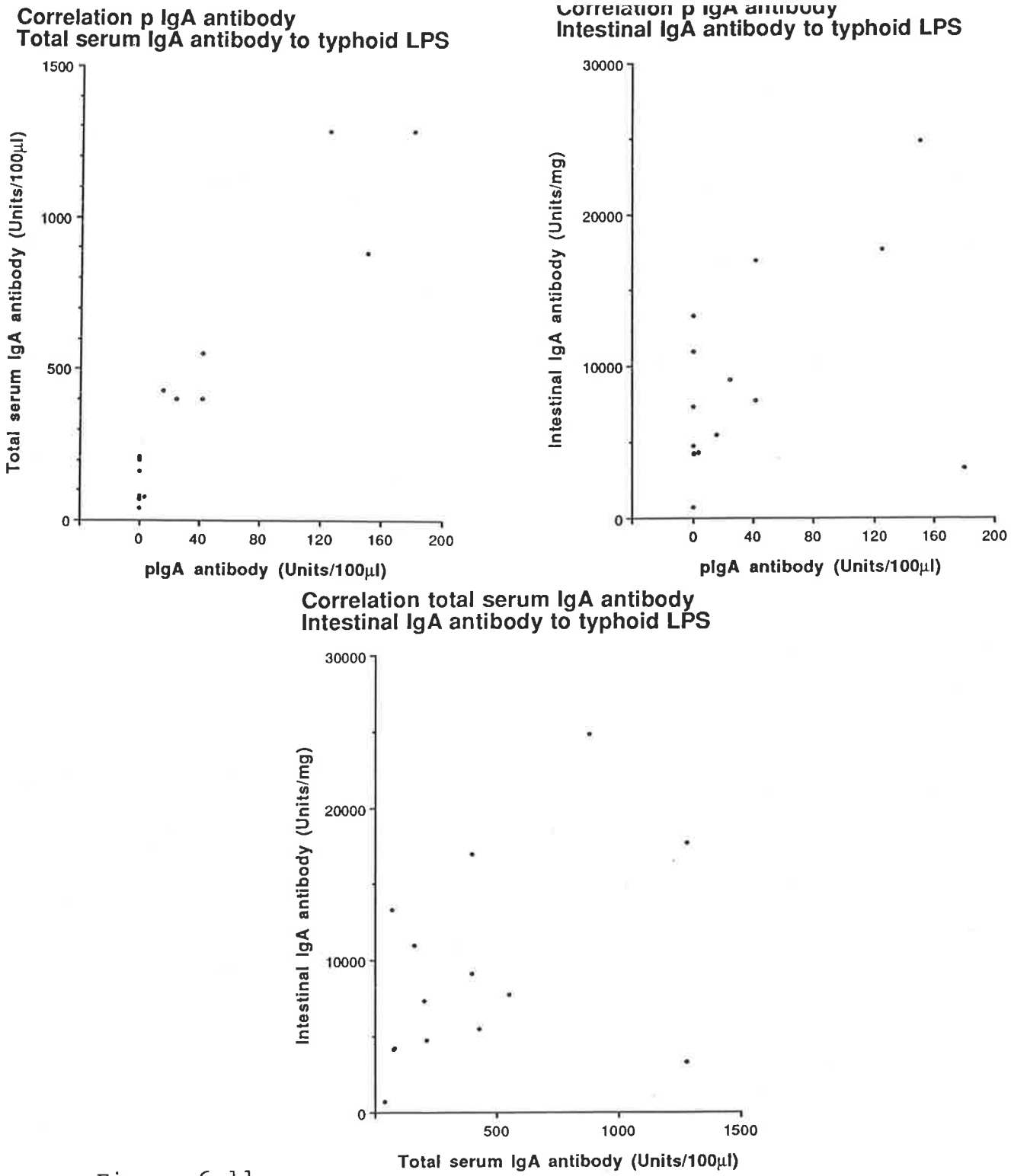


Figure 6.11

The relationship between serum and intestinal typhoid LPS-specific IgA antibody in the fourteen volunteers given the oral vaccine.

(a) Correlation between anti-typhoid serum pIgA antibody and total serum anti-typhoid IgA antibody

(b) Correlation between intestinal IgA and serum pIgA anti-typhoid antibody

(c) Correlation between intestinal IgA antibody and total serum IgA antibody to typhoid LPS.

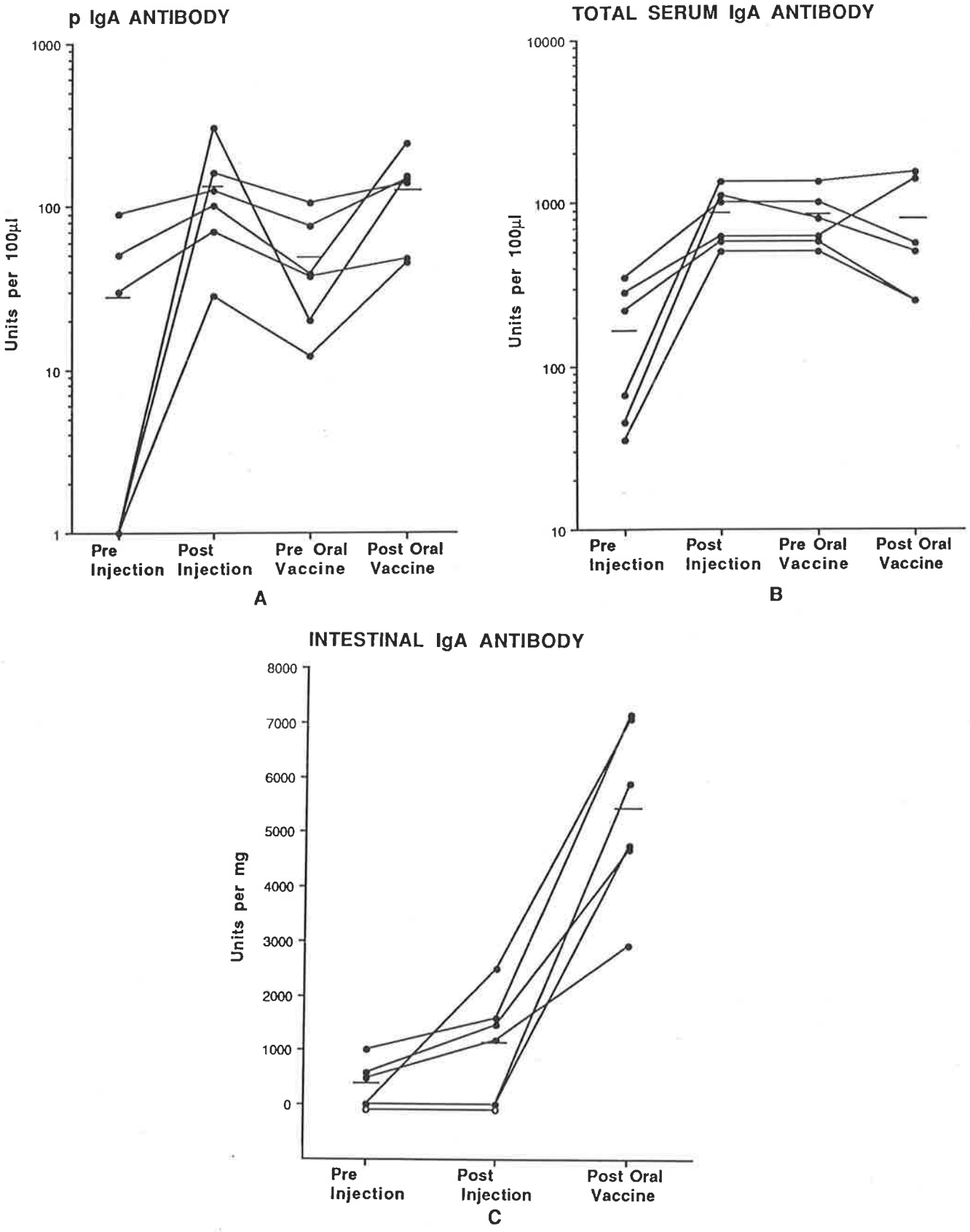


Figure 6.12

Typhoid LPS-specific IgA antibody response to parenteral vaccination followed by oral vaccine one month later. The group consisted of six volunteers. Horizontal bars indicate means

(a) pIgA antibody

(b) Total serum IgA antibody

(c) Intestinal IgA antibody

CHAPTER 7 - STUDIES IN PATIENTS WITH ALCOHOLIC LIVER DISEASE

I. INTRODUCTION

Alcoholic liver disease is associated with several abnormalities related to the immunoglobulin A system. In addition to high concentrations of serum IgA (Lee, 1965; Feizi, 1968; Wilson et al, 1969; Thompson et al, 1973; Iturriaga et al, 1977) alcoholic liver disease is also associated with the deposition of IgA in tissues such as the renal glomeruli (Nochy et al, 1976; Berger, Yaneva and Nabarra, 1978) and superficial skin capillaries (Kater et al, 1979; Swerdlow et al, 1983). The presence of IgA deposits in a continuous staining pattern along hepatic sinusoids in alcoholic liver disease has also been reported (Kater et al, 1979; Van de Wiel et al 1987 and 1988). Changes in the size and subclass of serum IgA occur. An increase in serum pIgA has been reported (André and André, 1976; Kutteh et al, 1982b; Sancho et al, 1982; Kalsi et al, 1983; Delacroix et al, 1983; Newkirk et al, 1983). Concerning IgA subclass both serum IgA1 and IgA2 are elevated but there appears to be a shift toward IgA2 (Van de Wiel et al, 1987). Serum secretory IgA levels are also increased (Thompson et al, 1973; Pelletier et al, 1982; Delacroix et al, 1982b) and finally circulating immune complexes containing monomeric IgA and pIgA have been observed (Sancho et

Another feature of alcoholic liver disease is the increased concentration of serum antibodies to bacterial components (Staun Olsen et al, 1983; Nolan et al, 1986). High levels of serum antibody to Escherichia coli (E.coli) have been reported (Bjørneboe et al, 1972; Prytz et al, 1973; Simjee et al, 1975; Staun Olsen et al, 1983). It has been suggested that this is due to intestinal antigens reaching the circulation in increased amounts. This could be due to the damaging effect of alcohol on the epithelium of the intestine (Draper et al, 1983). In addition impairment of secretion of antibody into the intestine has been demonstrated (Pelletier et al, 1982). This could result in excess absorption of antigen (Walker et al, 1974) and stimulation of the immune system.

In view of these reported abnormalities relating to the secretory immune system in patients with alcoholic liver disease and in view of the reports of high levels of serum pIgA in these patients, two studies were performed in patients with alcoholic liver disease. These studies were performed with the intention of obtaining further information regarding the immune system in these patients by firstly

examining levels of antigen specific pIgA antibody in these patients and secondly examining their capacity to respond to an immune stimulus delivered to the intestine. The relationship between intestinal IgA and serum IgA was also examined.

In the first study the SC binding assays were used to detect serum pIgA antibody to E.coli lipopolysaccharide (LPS) and to estimate the total serum pIgA level in a group of 14 patients with alcoholic disease and 8 normal controls.

In the second study the immune response of 11 patients with alcoholic liver disease to oral vaccination with the live attenuated oral typhoid vaccine Ty21a was evaluated. The immune response generated by this vaccine in intestinal fluid and serum was examined. The SC binding assays were used to detect typhoid LPS specific pIgA antibody and measure total serum pIgA. Intestinal and serum antibody to E.coli LPS was also measured in these 11 patients before vaccination, in an attempt to determine the relationship between serum and intestinal antibody to this intestinal organism.

II. FIRST STUDY:- SERUM POLYMERIC IgA ANTIBODY TO E.COLI LPS IN PATIENTS WITH ALCOHOLIC LIVER DISEASE.

1. Introduction

In this study the SC binding assays were used to detect pIgA antibody to E.coli LPS and to estimate the total serum pIgA in 14 patients with alcoholic liver disease and 8 normal controls.

2. Materials And Methods

a) Subjects

The patients with alcoholic liver disease had a history of alcohol abuse (daily consumption of greater than 80g a day for more than 5 years). They had clinical and biochemical signs of liver disease. The normal controls were healthy staff members. All sera were stored at -20^{o}C.

b) Assays

Assay For pIgA Antibody To E.coli LPS

The wells of microtitre plates were coated with E.coli LPS (Chapter 2, section V,3).

The assay for pIgA antibody to E.coli LPS was performed as previously described (Chapter 3, section II).

The coefficient of variation was 7.3% within the assay (n=6) and 13.1% between assays (n=11).

Assay For Total Serum pIgA

Total pIgA in serum was measured through its capacity to inhibit the binding of radiolabelled SC to IgM coated wells as described in Chapter 5, section II.

ELISA For Antibody To LPS

An ELISA using heavy chain-specific antibodies coupled to akaline phosphatase (Chapter 2, section V,3) was performed in parallel with the above mentioned assays, in order to detect total IgA antibody (monomeric and pIgA) agains E.coli LPS and to confirm the removal of IgM antibody by the immunoadsorbent step.

Measurement Of Total Serum IgA

Total serum IgA levels were measured by single radial immunodiffusion (Chapter 2, section V,4).

c) Statistical Analysis

The Mann Whitney U Test (two tailed) was used to analyse the differences between the patients and normal controls in respect of antibody to E.coli LPS and antibody concentrations.

3. Results

Detection Of Antibodies To **E.coli** LPS

pIgA antibody To E.coli LPS was detected in the IgM-depleted sera of patients with alcoholic liver disease (131±214 Units/100ul; pIgA) but could not be detected in the control sera (all <10 Units/100ul, Figure 7.1A). However, using the ELISA which detected both monomeric IgA and pIgA, antibodies to E.coli LPS could be detected in the sera from both patients and normal controls (Figure 7.1A). The ELISA antibody levels were significantly higher in the patients' sera than in the controls (1185±793 versus 56±19 Units/100ul; Figure 7.1A).

Total pIgA Levels

Total serum pIgA was higher in the patients than in the controls (488±333 and <120ug/ml; n=6 for controls, two of whom had undetectable pIgA levels, i.e. <20ug/ml; Figure 7.1B).

The patients also had elevated total serum IgA levels (6693±2464 ug/ml compared to 1925±384 ug/ml in the controls; Figure 7.1B).

The differences between patient and control sera with regard to IgA levels and IgA antibody were significant ($p<0.05$; Mann Whitney U Test, two tailed).

4. Discussion

Total serum IgA was elevated in these patients with alcoholic liver disease and the increase in total pIgA which was demonstrated was consistent with previous results using gel chromatography or sucrose density gradients (André and André, 1976; Kutteh et al, 1982b; Delacroix et al, 1983; Kalsi et al, 1983).

IgA antibodies to E.coli LPS were detected in both patients and controls using the ELISA, with the level in patients being significantly higher in keeping with previous reports (Bjørnoboe et al, 1972; Prytz et al, 1973; Simjee et al, 1975; Staun Olsen et al, 1983). Serum pIgA antibody to E.coli LPS could only be detected in patients and not in normal subjects. It is possible that the elevated pIgA antibody in these patients to LPS of the representative intestinal organism, E.coli reflects an increase in antibody production by lymphoid cells of mucosal origin. The alternative explanation may be the decreased clearance of pIgA in liver disease (Delacroix et al, 1983).

In an attempt to answer this question, both serum and intestinal IgA antibody to E.coli LPS were examined in a group of 11 patients with alcoholic

liver disease (Section III).

III. SECOND STUDY:-THE IMMUNE RESPONSE OF PATIENTS WITH ALCOHOLIC DISEASE TO THE ORAL TYPHOID VACCINE Ty21a AND THE RELATIONSHIP OF SERUM pIgA TO INTESTINAL IgA

1. Introduction

In this study the immune response of patients with alcoholic liver disease to the delivery of an antigenic stimulus to the intestine was assessed by measuring the IgA antibody response in serum and intestinal fluid following oral vaccination with Ty21a. A dose of the vaccine that reliably generates a measurable immune response in intestinal fluid and serum in normal subjects was used (see Chapter 6). The SC binding assays were used to measure anti-typhoid serum pIgA antibody and total serum pIgA. The relationship between anti-typhoid antibody in serum and intestinal fluid was examined.

Secondly as intestinal fluid was obtained from these patients an attempt was made to determine the relationship between serum and intestinal antibody to E.coli LPS in these patients by measuring serum pIgA antibody, total IgA antibody and intestinal IgA antibody to E.coli LPS in the samples obtained before typhoid vaccination.

Serum antibody to E.coli LPS and serum immunoglobulin levels in these 11 patients were compared to those in 13 normal controls.

2. Materials And Methods

Eleven patients with alcoholic liver disease consented to participate in this study. They all had a history of alcohol abuse (daily consumption of greater than 80g per day for more than 5 years). They had clinical and biochemical signs of liver disease. Nine of the 11 patients agreed to undergo intestinal intubation before and after vaccination. The controls were healthy laboratory staff.

a) Ty21a Vaccine

The vaccine was prepared as previously described (Chapter 2, section V,2a). Each dose contained $1x10^{11}$ freshly harvested viable organisms.

b) Vaccination Procedure

The patients were all inpatients of the Royal Adelaide Hospital who had been denied access to alcohol for at least 7 days before the vaccine was administered. After an 8 hour fast they were given 50ml of 2% sodium bicarbonate. The vaccine was administered 5 minutes later in 50ml of milk followed

by 100ml of distilled water. Each patient received 3 doses of vaccine, 48 hours apart.

c) Samples

Intestinal fluid and serum specimens were collected before vaccination and 3 weeks after vaccination (Chapter 2, section V,1). Two of the 11 patients did not consent to intestinal intubation.

d) Assays

Radioimmunoassays and Enzyme Linked Immunosorbent Assays

Polyvinyl plates containing 96 wells (COSTAR, Data Packaging Corp) were used for all the assays.

pIgA antibody to typhoid LPS and E.coli LPS was measured as described previously (Chapter 3, section II).

Total serum pIgA was measured as described in Chapter 5.

Intestinal IgA antibody and serum IgA, IgG and IgM antibody to typhoid LPS and E.coli LPS were measured by ELISA (Chapter 2, section V,3)

Plates were coated with 5ug/ml salmonella Ty2 LPS (SIGMA) that had been linked to methylated bovine serum albumin as previously described (Chapter 2, section V,3,a), or with 10ug/ml E.coli LPS as previously described (Chapter 2, section V,3,a). The ELISAs for typhoid LPS specific antibody and E.coli LPS specific antibody were performed according to the methods described in previous studies (see Chapter 2, section V,3). As a positive control the assay for typhoid LPS specific antibody included the serum of a convalescent typhoid patient with high antibody titre. In the assay for E.coli LPS specific antibody, serum from a patient with alcoholic liver disease with a high antibody titre to E.coli LPS was used as a positive control. The serum of a patient never exposed to typhoid was used as a negative control in the assay for typhoid specific antibody.

All samples from each individual were run in the same assay to allow comparison between antibody titres.

Immunodiffusion Assays

Total IgA immunoglobulin was measured by single radial immunodiffusion (Chapter 2, section V,4).

Statistical Analyses

See chapter 2, section V,5.

3. Results

a) Immunoglobulin Levels

As in the first study the concentration of total pIgA in the sera from the 11 patients with alcoholic liver disease was higher than in the sera of the 13 normal controls (795±746ug/ml and 39.3±26ug/ml respectively; Figure 7.2)

The patients also had elevated total serum IgA levels (5742±2513ug/ml in patients compared to 1329±711ug/ml in the controls; Figure 7.3).

These differences between patient and control sera with regard to pIgA levels and total serum IgA were significant (p=0.002).

b) Response To The Oral Typhoid Vaccine

After correction of the intestinal antibody titres for total secretory IgA content, 5 of the 9 patients from whom intestial fluid was obtained had a greater than 2 fold rise in intestinal IgA antibody to typhoid LPS (Figure 7.4). However, the group as a whole did not have a significant rise in intestinal antibody to typhoid after vaccination (Mean antibody

in units per mg pre vaccination 1944±1192; post vaccination 7764±9267; t=1.838, p=0.1033).

Only 5 of the 11 patients had a greater than 2 fold rise in serum pIgA antibody to typhoid LPS after vaccination (Figure 7.5). Intestinal fluid was obtained from 4 of these patients and all 4 had a greater than 2 fold rise in intestinal anti-typhoid IgA antibody. There was however no change in pIgA antibody in 1 of the patients who had an intestinal antibody response. The mean levels of pIgA antibody to typhoid pre and post vaccination were 116.1±125.2 and 623.6±1469.8 Units per 100ul respectively. The difference was not significant (t=1.2205, p=0.2503).

Of the 5 patients who had an intestinal IgA antibody response, 4 had a greater than 2 fold rise in serum IgA antibody to typhoid after vaccination (Figure 7.6). One of the 2 patients from whom only sera were obtained had a 6 fold rise in serum IgA antibody. This was the 5th patient who had a serum pIgA antibody response.

However, as only 5 of the total of 11 patients had a serum IgA antibody response to typhoid the differences pre- and post-vaccination in the group of 11 patients was not significant. (Serum IgA antibody

to typhoid pre- and post-vaccination 897±756 and 4844±10,304 units per 100ul respectively; t=1.329, p=0.2132).

In summary 5 of the patients had an intestinal antibody response, 4 of whom also had a response in serum pIgA antibody and total serum IgA antibody to the vaccine.

The levels of IgM antibody to typhoid in the 11 patients before and after vaccination were 2310±2186 and 4095±3810 units per 100ul respectively. This difference was not significant (t=1.873; p=0.0905). Three of the 11 patients had a greater than 2 fold rise in IgM antibody to typhoid.

In the IgG class the antibody level to typhoid rose in 7 of the 11 patients after vaccination and the difference between mean titres before and after vaccination (557±610 and 2520±3102 units per 100ul) were significant (t=2.257; p=0.0475).

c) Relationship Of Anti-Typhoid IgA Antibody In Intestinal Fluid To The Anti-Typhoid Serum pIgA And Total Serum IgA Antibody After Vaccination.

There was a significant correlation between the level of anti-typhoid serum pIgA antibody and the

total serum IgA anti-typhoid antibody after vaccination in the 11 patients (r_s=0.863, critical value of r_s at the 0.05 level of significance for the one tailed t-test = 0.564; Figure 7.7A).

There was no correlation between the level of intestinal IgA anti-typhoid antibody and the serum pIgA anti-typhoid antibody after vaccination in the 9 patients from whom both samples of intestinal fluid and serum were obtained (r_s=0.433 critical value of r_s at the 0.05 level of significance for the one tailed test = 0.600; Figure 7.7B). Neither was there a correlation between the intestinal IgA antibody and total anti-typhoid serum IgA antibody levels after vaccination (r_s=0.366 critical value of r_s at .05 level of significance = 0.600; Figure 7.7C).

d) Antibody to **E.coli** LPS

The results were similar to those observed in the first study. The patients had significantly higher levels of serum antibody to E.coli LPS than the 13 controls (Figure 7.8). Mean IgA antibody patients, 3304±3120, controls 361±388; IgG antibody patients, 1523±2875, controls 257±323 and IgM antibody patients 4974±3257 controls 1910±1357. Antibody units per 100ul respectively (Figure 7.8). These differences were significant (for serum IgA antibody p=0.002, IgG

and IgM antibody p=0.02, Mann Whitney U test). There was no significant difference between the antibody to E.coli LPS before and after vaccination with the oral typhoid vaccine (t=.577, p=0.57 for IgA; t=0.877, p=0.4006 for IgG; t=1.3177 p=0.2170 for IgM).

pIgA antibody to E.coli LPS was only found in the serum of patients 72±93.4 units per 100ul (Figure 7.9). There was no change in pIgA to E.coli after vaccination (mean 68.2±82.2 Units/100ul; not significant t=0.1347, p=.8955).

Intestinal IgA antibody to E.coli LPS was present in all 9 patients 5389±3784 units per mg. There was no increase in this antibody after typhoid vaccination 4391±2362 units per mg (t=0.986, p=-0.3526).

e) Relationship Of The Anti-**E.coli** LPS IgA Antibody In Intestinal Fluid To The Anti-**E.coli** Antibody In Serum

There was a significant correlation between the level of pIgA antibody to E.coli LPS and the total serum IgA antibody to E.coli LPS (r_s=.775 critical value of r_s at the 0.05 level of significance for the one tailed t-test = 0.564; Figure 7.10A). There was no correlation between the level of serum pIgA antibody to E.coli LPS and the intestinal IgA antibody to

E.coli LPS (r_s=.004 critical value of r_s at the 0.05 level of significance for the one tailed t-test = 0.600; Figure 7.10B). In addition there was no correlation between intestinal IgA and total serum IgA antibody against E.coli LPS (r_s=.229, critical value of r_s at the 0.05 level of significance =0.600; Figure 7.10C).

4. Discussion

In this second study the 11 patients with liver disease had high levels of serum IgA and a pronounced elevation in serum pIgA. They had significantly higher levels of serum IgA antibody to E.coli LPS than normal controls. As in the previous study (Section II) E.coli LPS specific pIgA antibody was only detected in the patients sera. Although E.coli LPS specific intestinal IgA antibody was detected in 9 patients who consented to intestinal intubation, there was no correlation between the level of intestinal antibody and serum pIgA antibody to E.coli LPS. Despite having high levels of total serum IgA and total serum pIgA and high levels of serum antibody to E.coli LPS these patients had an impaired immune response to the oral typhoid vaccine. The reasons for these findings and their possible significance will now be dicussed.

The elevated pIgA concentration is consistent with previous reports where pIgA was measured using gel chromatography or sucrose density gradients (André and André, 1976; Kutteh et al, 1982b; Delacroix et al, 1983; Kalsi et al, 1983). Newkirk et al (1983) used a SC binding assay which also demonstrated elevated pIgA in patients with alcoholic liver disease. As in the previous study (Chapter 7, section II) the normal controls had serum pIgA levels under 120ug per ml.

These changes in IgA characteristics observed in the two studies in patients with alcoholic liver disease could be due to a number of factors, some related to the alcohol aetiology and some related to the liver damage. There is evidence for both increased synthesis and decreased catabolism of IgA in patients with alcoholic liver disease.

Alcoholic patients have abnormalities which disturb the integrity of the gastrointestinal mucosa which could promote the absorption of materials which are normally excluded (Lai et al, 1976; McLelland et al, 1976; Draper et al, 1983). The increased antigenic load could result in enhanced stimulation of the immune system promoting an increased secretion of IgA antibody. Increased in vitro synthesis of both monomeric and pIgA by peripheral blood lymphocytes

obtained from patients with alcoholic liver disease has been demonstrated (Kalsi et al, 1983). This could explain the increase in both pIgA and total IgA observed in the present study. It has also been suggested that the decreased integrity of the mucosa could cause influx of IgA into the circulation (Underdown and Schiff, 1986). These patients have decreased intestinal secretory IgA (Pelletier et al, 1982) and increased serum secretory IgA (Thompson et al, 1973; Pelletier et al, 1982) and it is possible that defects in the trans enterocyte transport of IgA into the intestine could lead to increased leakage of pIgA into the circulation thus causing the increase in serum pIgA observed in the present studies.

In addition to factors that could lead to excess IgA production, a reduction in the capacity of the liver to clear IgA (Delacroix et al, 1983) might explain the findings of elevated serum pIgA in these patients. The liver is an integral part of the enteric mucosal immune system (Kleinman et al, 1982; Bienenstock and Befus, 1983) and has a major role in the metabolism of IgA (Conley and Delacroix, 1987). Elevated serum pIgA has been observed in various liver disorders other than alcoholic liver disease such as biliary cirrhosis (Delacroix et al, 1983; Newkirk et al, 1983). The reasons for this are unclear.

In various animal species pIgA is selectively transported from the blood into bile by SC located on the sinusoidal surface of hepatocytes (Orlans et al, 1978; Jackson et al, 1978; Lemaitre-Coelho et al, 1978; Orlans et al, 1979; Socken et al, 1979; Fisher et al, 1979). This SC dependent transport of IgA into bile via hepatocytes is less important in man. In humans only 0.5mg/kg body weight per day of pIgA is transported into bile compared to 30mg/kg body weight per day in the rat (Conley and Delacroix, 1987) and there is no increase in serum pIgA when biliary obstruction occurs (Delacroix et al, 1983). However, patients with parenchymal liver disease do have a prolonged serum half life of pIgA (Delacroix et al, 1983) suggesting that despite the lack of significant hepatobiliary transport, the liver does play a role in the clearance of serum pIgA in humans. Studies have shown that human hepatocytes bind IgA, preferentially pIgA (Hopf et al, 1978). The identity of this receptor has not been established as the detection of SC by some investigators (Hsu and Hsu, 1980; Foss Bowman et al, 1983) has not been confirmed by others (Delacroix and Vaerman, 1983; Hopf et al, 1978; Nagura et al, 1981). A hepatic binding protein on the cell surface of hepatocytes which binds asialoglycoproteins including IgA has been proposed as a possible

mechanism for IgA clearance (Stockert et al, 1982) and Tomana et al, (1988) have been able to demonstrate the endocytosis and catabolism of IgA mediated by the asialoglycoprotien receptor in human liver tissue. Another potential mechanism of IgA clearance which may be affected are the Kupfer cells of the reticulo endothelial system (Conley and Delacroix, 1987). In mice IgA immune complexes injected intravenously can be located in Kupfer cells (Rifai and Mannik, 1984). Finally, endothelial cells lining the sinusoids may have a role in IgA clearance (Nagura et al, 1981). Thus although the precise mechanism of IgA metabolism awaits clarification impaired liver function seems to particularly affect the clearance of pIgA.

In this study antibody to E.coli LPS was present in the serum of patients and controls. The patients with alcoholic liver disease had significantly higher levels of IgA antibody to E.coli LPS than the controls. This is in keeping with previous reports (Bjørneboe et al, 1972; Prytz et al, 1973; Simjee et al, 1975; Staun Olsen et al, 1983). As in the previous study (Section II) serum pIgA to E.coli LPS was only detected in patient sera. The presence of high levels of anti E.coli pIgA antibody may be due to increased production of pIgA by the IgA cells in the lamina propria in response to the entry of components

of these intestinal organisms though the disturbed intestinal epithelium. In addition it has been suggested that the high levels of serum antibody to E.coli LPS are due to the failure of the liver to extract circulating antigens and gut derived endotoxin (Staun Olsen et al, 1983). Endotoxaemia of enteric origin is common in these patients with alcoholic liver disease (Nolan, 1975). Although the portal blood normally carries small amounts of absorbed endotoxin it is usually removed and detoxified by the liver (Jacob et al, 1977). Liver injury impairs removal and detoxification leading to spillover of LPS into the system circulation (Nolan, 1981). Endotoxins are potent mitogens and so the high antibody levels could be due to enteric antigens that bypass the liver to reach the antibody forming organs (Nolan, 1986). The presence of high levels of IgG and IgM antibody to E.coli LPS in the present study would suggest stimulation of a systemic immune response. In addition in the present study there was no correlation between intestinal IgA antibody to E.coli and the serum pIgA to E.coli. However, there was a correlation between serum pIgA antibody to E.coli and total serum IgA antibody to E.coli in these patients. This would suggest that the elevated pIgA antibody to E.coli was more likely to have originated in the systemic compartment of the immune system in these patients.

It was interesting to observe the results of vaccination via the oral route in these patients. Compared to normal volunteers (Chapter 6) the mucosal immune response in these patients was depressed. Normal volunteers given 3 doses of 10^{11} organisms consistently produce intestinal antibody against typhoid LPS. In this study only 5 of the 9 patients had an intestinal antibody response. Of the 5 who had an intestinal response only 4 showed a rise in serum anti-typhoid IgA antibody. Alcohol has various, mostly suppressive effects on the immune system (Macgregor, 1986) and this may be why the immune response was depressed. Although the patients had high levels of total serum pIgA a serum pIgA response to the vaccine was only observed in 5 patients. Other studies in healthy volunteers (Chapter 6; Mascart Lemone et al, 1987) have shown the pIgA antibody response to vaccination in normal subjects occurs early, around 10 to 14 days after vaccination and is short lived. It is conceivable that the serum pIgA response to the vaccine was missed in these patients as samples were obtained 3 weeks after vaccination. Although a secretory IgA antibody response occurred in a small proportion of the patients the serum IgG antibody levels to the typhoid organism rose. It raises the possibility that the antigen may have

reached the systemic circulation to stimulate the production of IgG antibodies.

In summary the group of patients with alcoholic liver disease had high levels of serum IgA and a pronounced elevation in pIgA together with high levels of serum antibody to E.coli LPS. There was no correlation between the intestinal and serum pIgA antibody to E.coli LPS. The patients had an impaired immune response to an antigen delivered into the intestine. Despite the high levels of serum pIgA in these patients there was no correlation between the level of intestinal anti-typhoid antibody and the serum pIgA anti-typhoid antibody. These findings indicate that serum pIgA antibody does not reflect antibody produced in the intestine in patients with alcoholic liver disease.

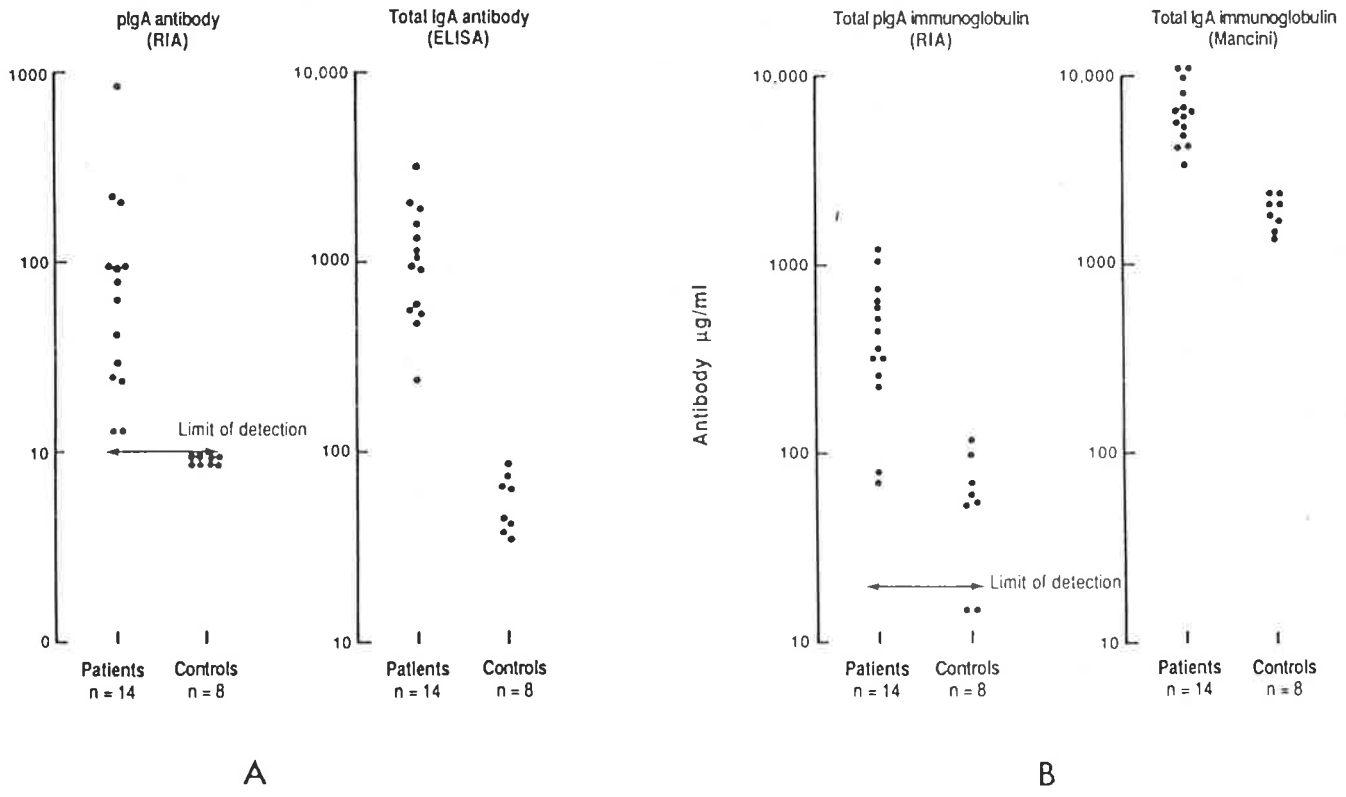


Figure 7.1

IgA antibody and immunoglobulin levels in sera from patients with alcohol induced liver disease and control subjects. A: Antibody to E.coli LPS. pIgA antibody (left), total IgA antibody (monomeric plus pIgA) (right). Lower limit of detection 10 Units of antibody per 100ul. B: Immunoglobulin levels. pIgA (left), total IgA (monomeric plus pIgA) (right). Only concentrations above 20ug/ml pIgA were detected. pIgA could not be detected in 2 of 8 control sera.

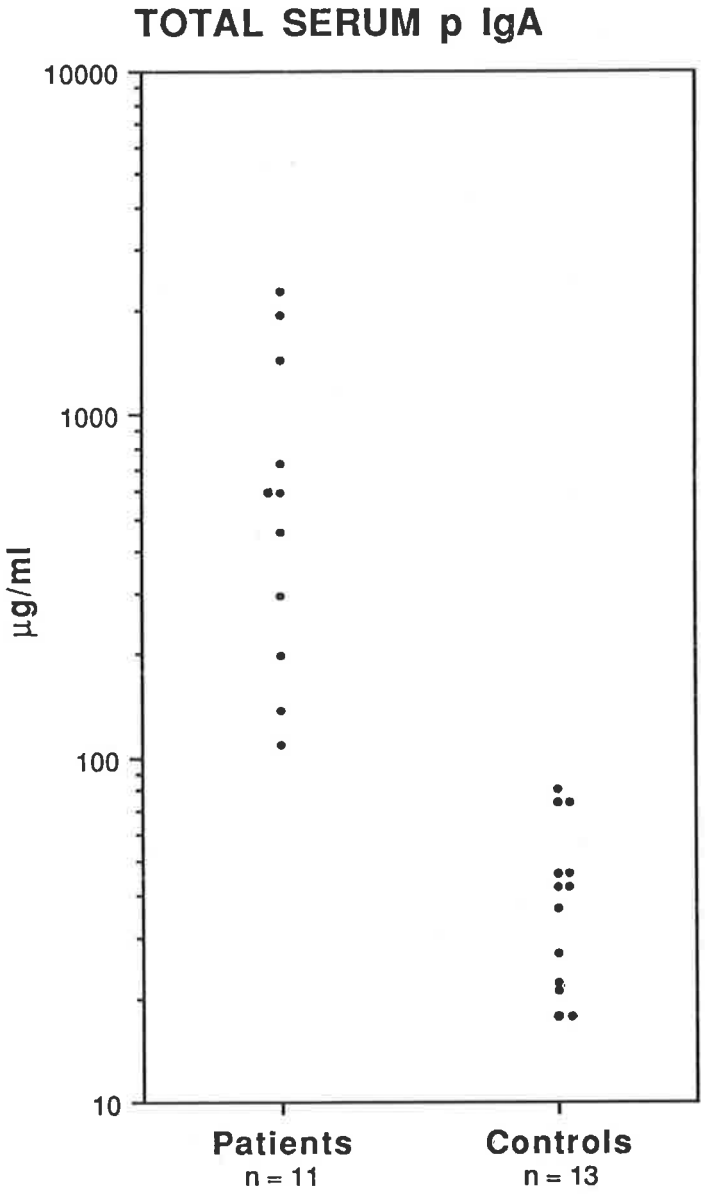


Figure 7.2

Total pIgA immunoglobulin levels in the 11 patients with alcohol induced liver disease who were given the oral typhoid vaccine and in 13 control subjects.

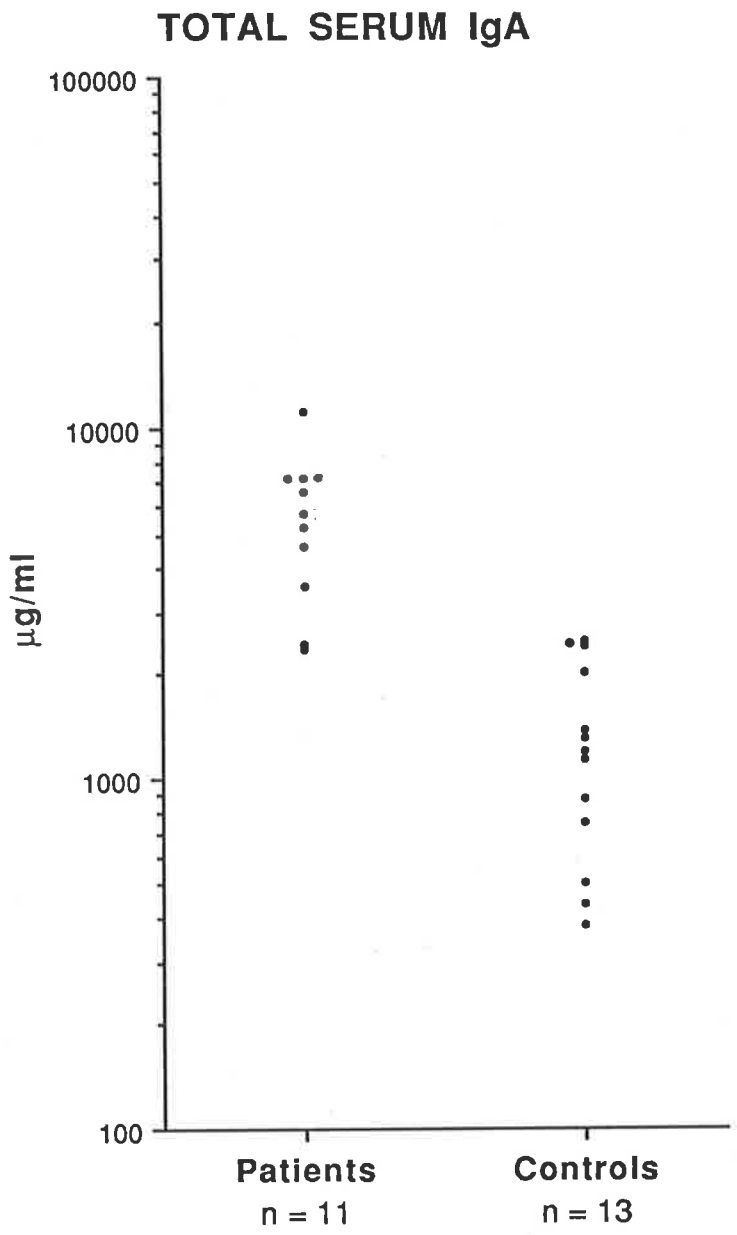


Figure 7.3

Total IgA immunoglobulin levels (monomeric plus pIgA) in the 11 patients with alcohol induced liver disease who were given the oral typhoid vaccine and in 13 control subjects. Total IgA was measured by single radial immunodiffusion.

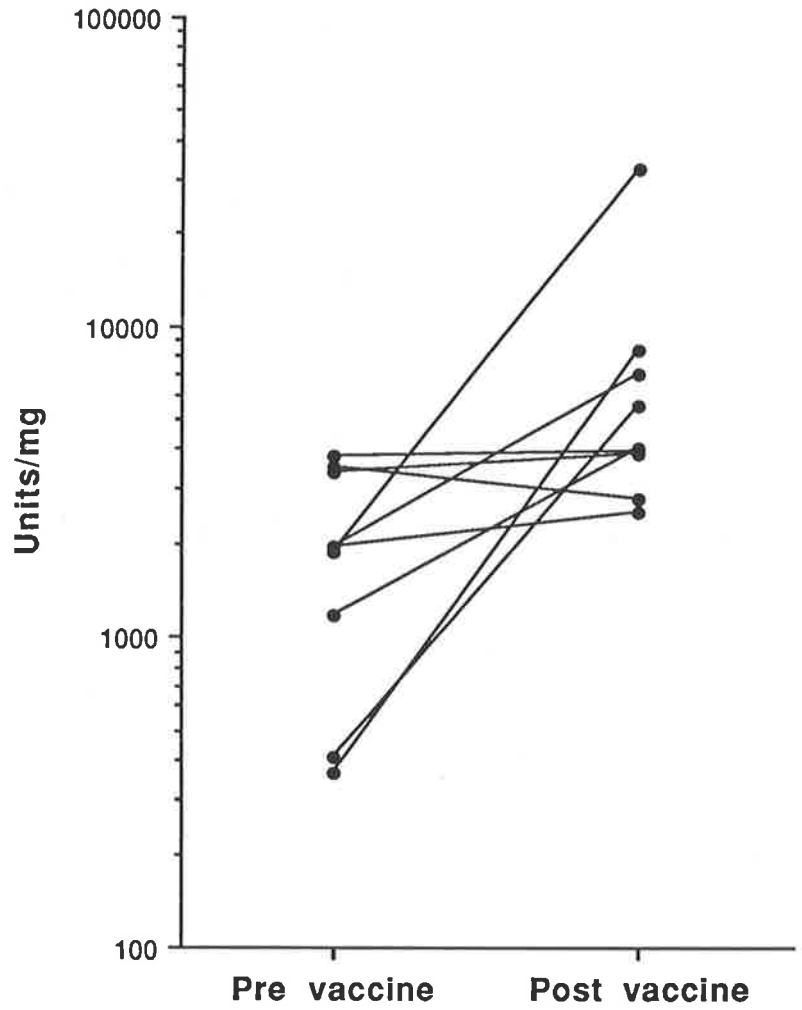


Figure 7.4

Intestinal IgA antibody to S.typhi LPS pre-vaccination and 3 weeks post-vaccination in 9 patients with alcohol induced liver disease given the oral typhoid vaccine Ty21a.

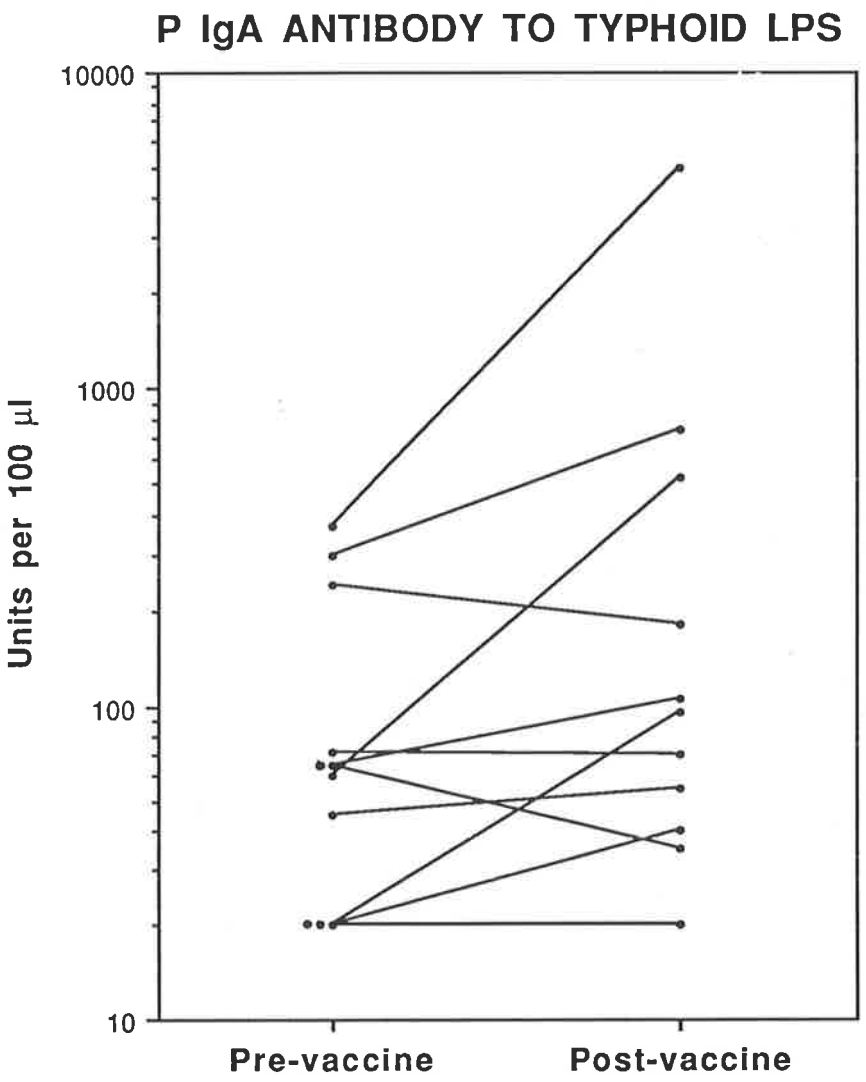


Figure 7.5

Typhoid-LPS specific pIgA antibody pre-vaccination and 3 weeks post-vaccination in the 11 patients with alcohol induced liver disease given the oral typhoid vaccine Ty21a.

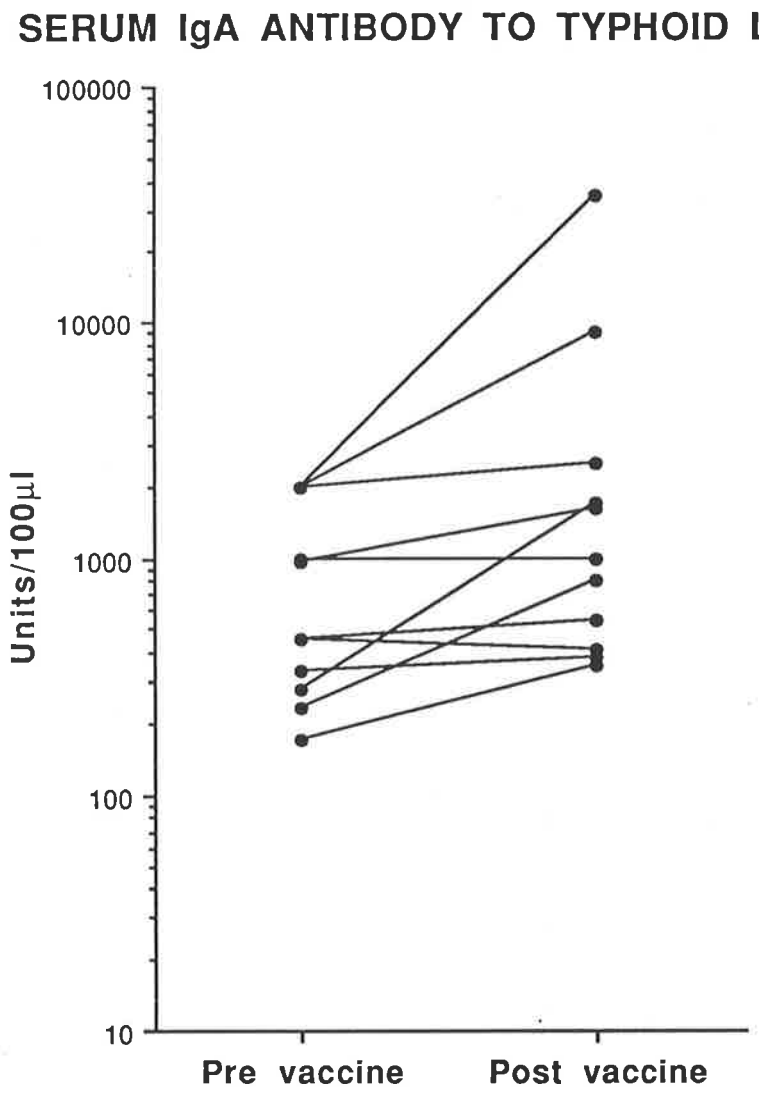


Figure 7.6

Typhoid-LPS specific total IgA antibody (monomeric and pIgA) pre-vaccination and 3 weeks post-vaccination in the 11 patients with alcohol induced liver disease given the oral typhoid vaccine Ty21a.

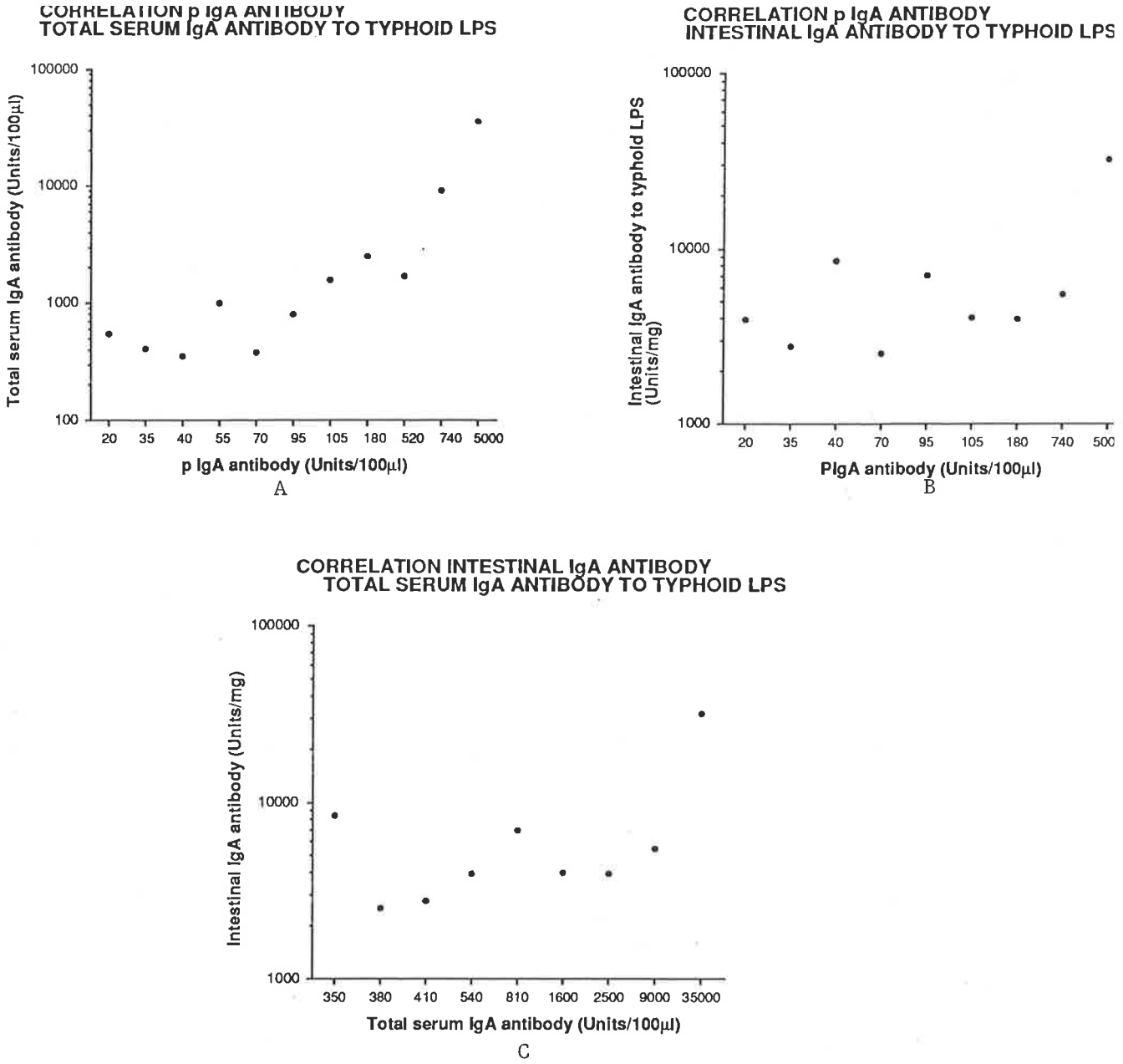


Figure 7.7

The relationship between serum and intestinal typhoid LPS-specific IgA antibody in the patients with alcohol induced liver disease who received the oral typhoid vaccine.

A. Correlation between anti-typhoid serum pIgA antibody and total serum anti-typhoid IgA antibody after vaccination (n=11).

B. Correlation between intestinal IgA antibody and serum pIgA anti-typhoid antibody (n=9).

C. Correlation between intestinal IgA antibody and total serum IgA antibody to typhoid LPS (n=9). (Intestinal fluid samples were obtained in 9 of the 11 patients).

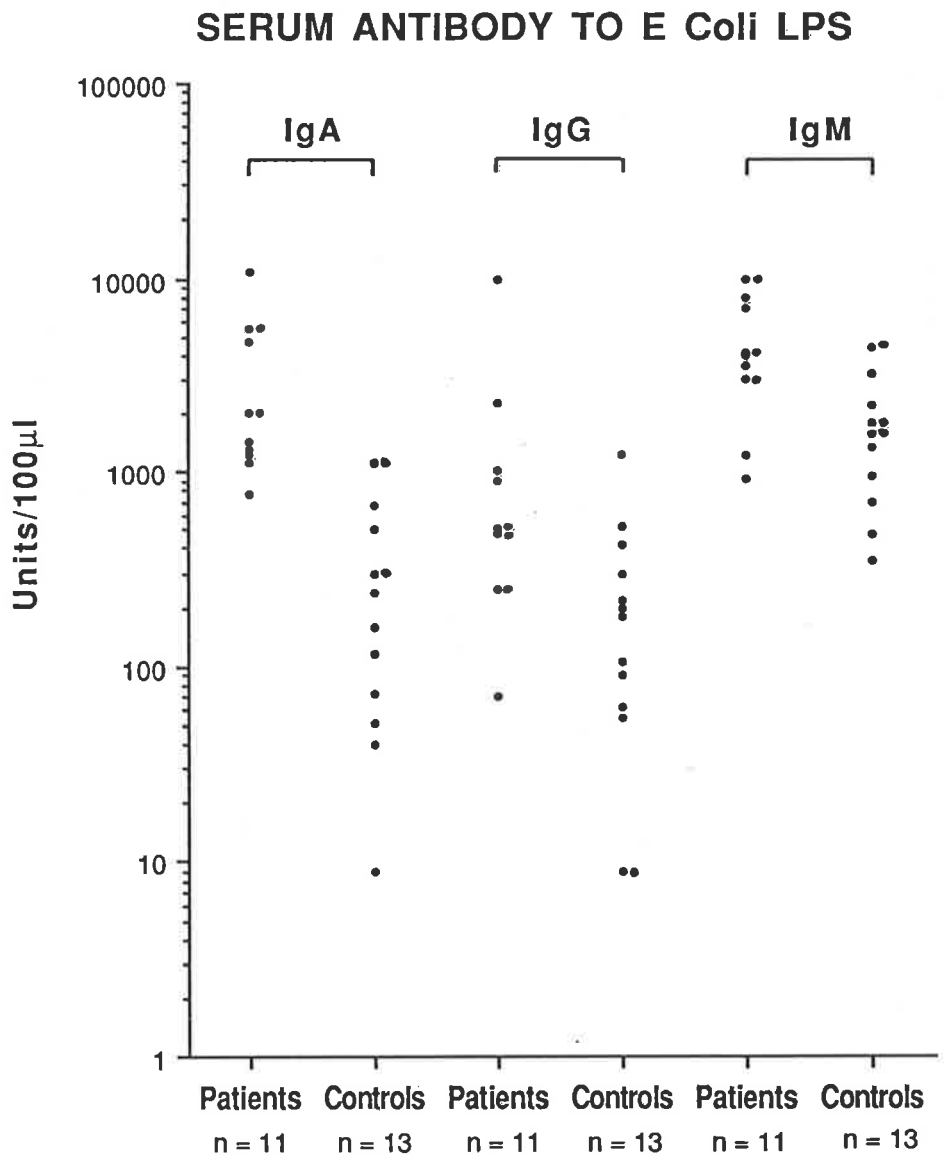


Figure 7.8

Serum antibody to E.coli LPS in patients with alcohol induced liver disease (n=11) and control subjects (n=13). IgA antibody (left), IgG antibody (centre), IgM antibody (right).

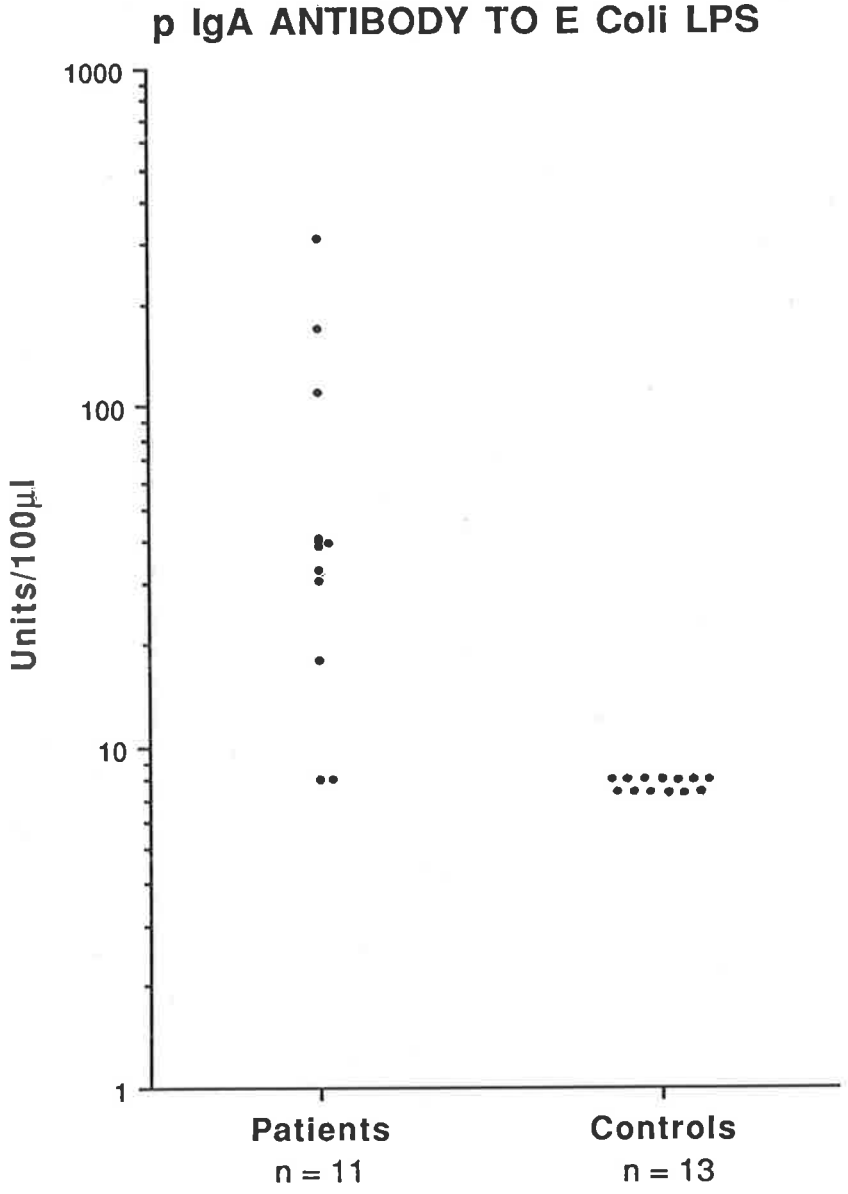


Figure 7.9

Serum pIgA antibody to E.coli LPS in patients with alcohol induced liver disease (n=11) and control subjects (n=13).

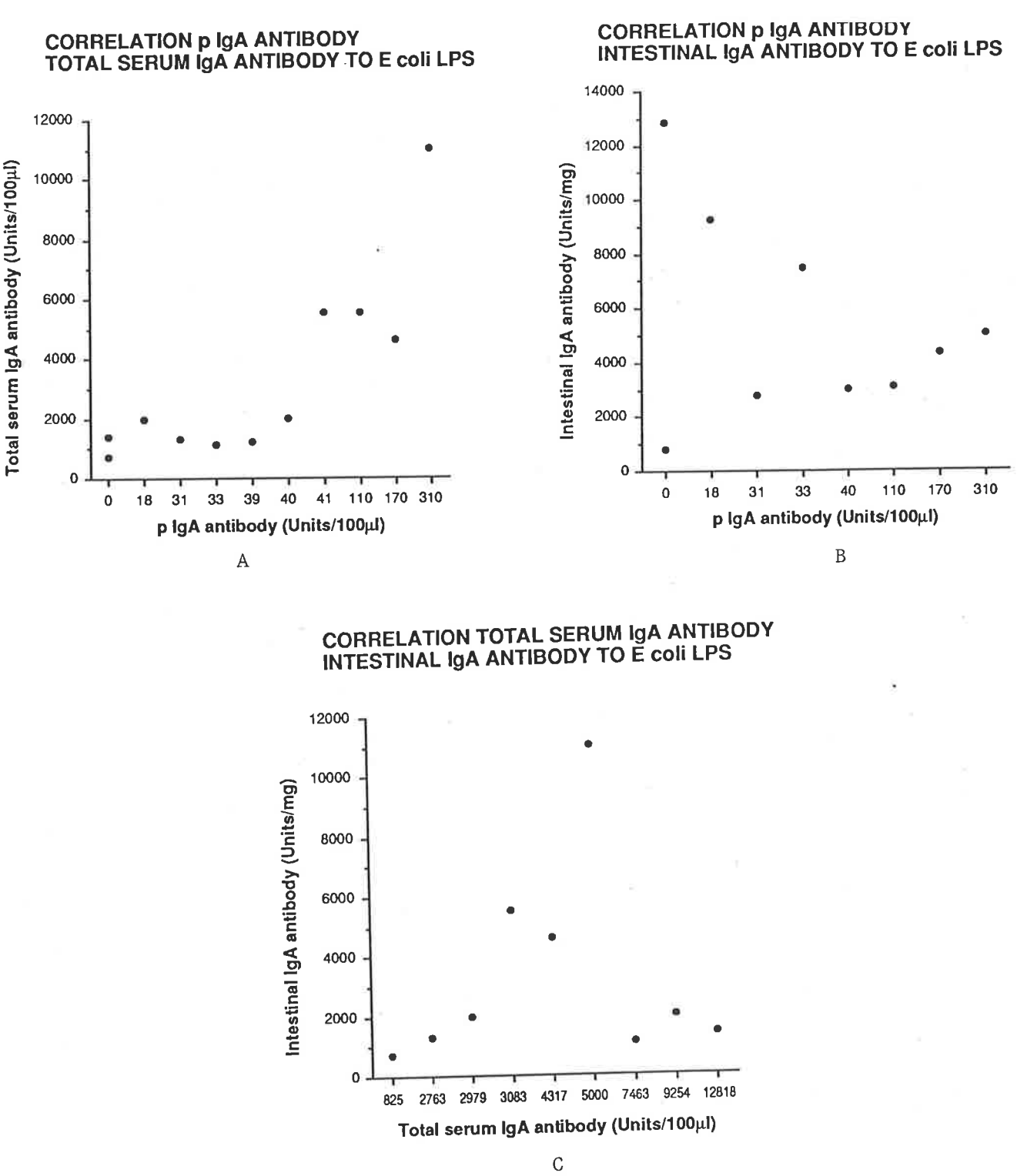


Figure 7.10

The relationship between serum and intestinal E.coli-specific antibody in patients with alcohol induced liver disease.

A. Correlation between total serum IgA antibody (monomeric plus pIgA) and serum pIgA antibody (n=11).

B. Correlation between intestinal IgA antibody and serum pIgA antibody (n=9).

C. Correlation between intestinal IgA antibody and total serum IgA antibody (n=9).

CHAPTER 8 - POLYMERIC IgA ANTIBODY IN DISEASE

I. INTRODUCTION

It has been suggested that abnormalities in the secretory immune system may play a role in the pathogenesis of some diseases such as Coeliac disease, IgA nephropathy and Crohn's disease. The SC binding assay was utilized to examine antigen specific pIgA in patients with these diseases in order to extend the study of the relationship of pIgA antibody in serum to intestinal immune responses.

In addition the serum pIgA response to a defined stimulus in the form of Campylobacter infection in otherwise healthy individuals was examined.

II. POLYMERIC IgA ANTIBODY TO GLIADIN IN THE SERUM OF PATIENTS WITH COELIAC DISEASE

1. Introduction

Coeliac disease (gluten sensitive enteropathy) is a disease in which the small intestinal mucosa of susceptible persons is damaged after eating gluten containing foods. The lesion heals when gluten is removed from the diet but reappears on its reintroduction (Falchuk, 1979).

In a study of both serum and intestinal antibody in coeliac disease, LaBrooy et al (1986) described a

relatively rapid fall in serum IgA antibody to gluten and gliadin derived peptides when patients went onto a gluten free diet, while intestinal IgA antibody to the same proteins remained elevated. This divergence was presumed to reflect the different origins of serum and intestinal IgA antibody.

In this study the SC binding assays were used to measure both specific pIgA anti-gliadin antibody as well as total pIgA in sera of patients with coeliac disease. The relationship of the serum pIgA antibody to dietary gluten and to the anti-gliadin IgA antibody in intestinal fluid was also examined.

2 Materials And Methods

a) Subjects

The patients studied were those in whom an unequivocal diagnosis of coeliac disease had been made. In adults the diagnosis was made on the grounds of a biopsy showing histological evidence of sub-total villous atrophy and a clear response to a gluten-free diet (cessation of diarrhoea and weight gain). In the children the diagnosis was made according to the criteria of the European Society of Paediatric Gastroenterology and Nutrition (McNeish et al, 1979). They were studied while on a diet containing gluten and after they had been on a gluten free diet

for at least 6 months.

The mean age of the adults was 46 years (range 22 to 78) and the children was 7 years (range 2 to 15).

Both serum and intestinal fluid were obtained from 7 children at presentation and after gluten exclusion. Serum alone was obtained from 25 patients (23 adults, and 2 children), 12 samples from subjects ingesting gluten and 17 from subjects on a gluten free diet (8 were paired samples from 4 adult subjects on and off gluten).

b) Samples

The intestinal fluid was obtained through a tube attached to a Watson paediatric intestinal biopsy capsule which was positioned at the duodeno-jejunal flexure with the aid of fluoroscopy. Only intestinal fluid with a pH greater than 7 was used in these studies. The samples were collected in containers containing 2mM phenyl-methyl sulphonyl fluoride (PMSF) a protease inhibitor (Hohmann et al, 1983) and frozen at -70^{o}C until the assays were performed.

All sera were stored at -20^{o}C until assayed.

c) Assays

Micro titre plates with 96 wells (Costar, Data Packaging Corp., Cambridge, Mass., USA) were used for the assays. Gliadin, was attached to the plates by dissolving it in 70% ethanol at a concentration of 5ug/ml, adding 100 ul per well and evaporating the solvent to dryness overnight at room temperature (Chapter 2, section V,3a,iii).

Radioimmunoassay For pIgA Antibody To Gliadin

The assay for pIgA antibody to gliadin was performed as described in Chapter 3 (Section II).

The intra assay coefficent of variation and inter assay coefficent of variation was 16.7% and 17.8% respectively (based on 7 assays).

Radioimmunoassay For Total Serum pIgA

Total serum pIgA was measured as described in Chapter 5 (Section II).

Enzyme Linked Immunosorbent Assay (ELISA) For Antibody To Gliadin In Serum And Intestinal Fluid

The assay used was an ELISA described previously (Hohmann et al, 1983). 100ul aliquots of serum or intestinal fluid were serially diluted in gliadin coated microtitre plates and then incubated for 4

hours at 4°C for serum or for 2 hours at 20°C for intestinal fluid. In the case of intestinal fluid the incubation was carried out in the presence of 2mM phenyl-methyl sulphonyl fluoride (PMSF) a protease inhibitor which improves the sensitivity of the assay (Hohmann et al, 1983). IgA and IgM antibody binding to the wells was detected using heavy chain specific antisera coupled to alkaline phosphatase (Kirkegaard Perry Laboratories) followed by addition of substrate (Sigma para nitro phenyl phosphate).

Results are expressed as units of antibody per 100ul which represents the reciprocal of the dilution of sera or intestinal aspirate which gives an O.D.405 of 0.15 (Hohmann et al, 1983). As the immunoglobulin content of intestinal fluid is variable the intestinal antibody results are expressed in terms of units of antibody per mg of immunoglobulin in the sample.

Immunodiffusion Assays

Total immunoglobulin in the IgA (monomeric and pIgA) and IgM class was measured by single radial immunodiffusion (Chapter 2, section V,4)

3. Results

In the subjects from whom only serum was obtained, 11 of the 12 subjects on a diet which

contained gluten had specific pIgA antibody to gliadin. In contrast only 3 of the 17 subjects who had excluded gluten from their diet had pIgA antibody to gliadin in serum (Figure 8.1). In 3 of the 4 subjects from whom paired samples on and off gluten were obtained, pIgA antibody against gliadin was detected while they were ingesting gluten and not after they eliminated it from their diet (Figure 8.1). The total serum IgA anti-gliadin antibody (monomeric and pIgA) was significantly higher in the group of subjects ingesting gluten compared to those on a gluten free diet and it fell in the 4 subjects from whom paired samples were obtained (Figure 8.1). The level of total serum pIgA was higher in each of these 4 subjects while they were ingesting gluten although the difference between the total pIgA immunoglobulin and total IgA levels between the 2 groups of patients on and off gluten was not significiant (Figure 8.2). However, the levels of total pIgA immunoglobulin in the sera of patients on gluten were significantly higher than the levels of total pIgA found in the normal subjects studied in chapter 7, section II ($u=11$: $p<0.02$).

Considering the 7 children, from whom paired samples of intestinal fluid and serum were obtained, one of them was IgA deficient in both serum and

intestinal secretions. pIgA antibody to gliadin was detected in 3 of the other 6 while on gluten. No pIgA antibody to gliadin was detected in serum after exclusion of dietary gluten (Figure 8.3). Total serum anti gliadin IgA antibody fell (Figure 8.3). Intestinal IgA antibody to gliadin did not change (Figure 8.3).

4. Discussion

This study demonstrates the presence of pIgA antibody to gliadin in the serum of patients with coeliac disease particularly while they ingest gluten (14 of 18). In only 3 of the 23 subjects studied while on a diet which was meant to be gluten free was pIgA antibody to gliadin detectable. It is possible that these 3 patients were less than ideally compliant with their diet; their total anti-gliadin antibody as measured using the ELISA also revealed elevated levels (LaBrooy et al, 1986). The findings are in agreement with those of Mascart-Lemone et al (1988), who measured polymeric antigliadin antibody by sucrose density gradient ultracentrifugation. Increased serum antigliadin pIgA antibody was only found in children with coeliac disease who were ingesting gluten.

One of the central questions that prompted this study was the possibility that polymeric IgA antibody

to gliadin in serum could relate to intestinal IgA antibody to gliadin. The findings in the 6 children in whom suitable samples of intestinal fluid and serum were obtained on and off gluten in this study, taken in conjunction with previous findings by LaBrooy et al (1986) in which intestinal antibody to gliadin tended to remain elevated when patients went onto a gluten-free diet, suggest that no such direct relationship exists. Indeed, there was a closer relationship between serum pIgA anti-gliadin antibody and total IgA antibody to gliadin in serum than to intestinal IgA anti-gliadin. This would tend to suggest that the polymeric IgA found in serum is not merely a fraction spilling back into serum from the intestinal lamina propria lymphocytes synthesising polymeric IgA but has other origins, or that there are factors other than the rate of this synthesis that have an impact on serum levels of polymeric IgA.

This present study also revealed that there were increased levels of total pIgA in patients with coeliac disease compared to normal subjects who have total pIgA levels less than 120ug/ml (Chapter 7, section II, III; Figure 7.1 and 7.2). Although the levels of total pIgA in patients with coeliac disease on a diet containing gluten appeared to be higher than those in patients on a gluten-free diet, this did not

reach significance. This may reflect the limitations both of numbers and the lack of information regarding the degree of mucosal recovery at the time of sampling in patients on a gluten-free diet. The results would suggest that increased levels of total pIgA in coeliac disease may make a small contribution to the increased gliadin-specific pIgA in serum.

III. SERUM POLYMERIC IgA IN PATIENTS WITH IgA NEPHROPATHY AND THE IMMUNE RESPONSE TO ORAL TYPHOID VACCINATION

1. Introduction

IgA nephropathy is a form of glomerulonephritis in which the diagnostic hallmark is the finding of mesangial deposits of IgA (Berger and Hinglais, 1968). The disease is usually found in young adults presenting with gross or microscopic haematuria. Although it is generally thought to result in only mild renal impairment it may progress to chronic renal failure (Clarkson et al, 1977). The manner in which IgA is deposited in the kidneys and the role of IgA in the pathogenesis of the disease is still uncertain. As IgA is the principal immunoglobulin in secretions and since episodes of gross haematuria and exacerbations of the disease frequently occur in association with infections of the respiratory and gastrointestinal tract, it has been suggested that the secretory immune

system and serum pIgA have a role in the pathogenesis of the disease.

Some of these patients have elevated levels of serum IgA (Clarkson et al, 1977; Newkirk et al, 1983). High serum levels of pIgA (André et al, 1980; Lopez Trascasa et al, 1980; Newkirk et al, 1983; Valentjin et al, 1984) and increased rates of synthesis of pIgA by circulating lymphoid and tonsillar cells from patients with this disease have also been reported (Egido et al, 1982 and Egido et al, 1984).

Several findings suggest that the IgA deposited in the glomeruli is pIgA. The IgA deposits are capable of binding SC and stain with anti J chain antibodies (Béne et al, 1982; Tomino et al, 1982; Donini et al, 1983; Komatsu et al, 1983). In addition elution studies show that the IgA deposited in the glomeruli is polymeric (Tomino et al, 1982).

André et al (1980) reported that IgA_2 was found to a greater extent than IgA_1 in the mesangium and proposed a mucosal origin of the deposits in view of the predominance of IgA_2 in the mucosa and predominance of monomeric IgA_1 in the circulation. In contrast to this, recent studies have suggested that the IgA deposits are almost exclusively of the IgA_1

sub-class (Conley et al, 1980; Tomino et al, 1982; Valentjin et al, 1984). Valentjin et al (1984) however, found that the IgA_1 in the mesangial deposits was capable of binding SC and contained J chain suggesting that it was polymeric. Therefore the link between stimulation of the mucosal immune system and the deposition of mesangial IgA is not straightforward.

The role of antigenic stimulation has been investigated by examining the immune response to vaccination. There have been studies in humans which suggest that these patients respond to parenteral immunization with an augmented IgA antibody response. An augmented IgA response to inactivated mumps vaccine (Pasternak et al, 1986) and influenza HA virus (Endoh et al, 1984) has been reported. More recently an enhanced antibody response to the oral polio vaccine has been reported (Leinniki et al, 1987).

The aim of the study was firstly to measure the total serum pIgA in a group of patients with IgA nephropathy and secondly to investigate the immune response of these patients to oral vaccination with the live attenuated bacterial vaccine Salmonella typhi Ty21a focussing on the serum pIgA response.

2. Materials And Methods

a) Subjects

Five patients with IgA nephropathy (confirmed by renal biopsy and histopathology) gave informed consent for this study which had the approval of the human ethics committee of the Royal Adelaide Hospital. One subject was a recently-diagnosed patient with normal renal function, the other 4 had varying degrees of renal impairment.

b) Vaccine

The organism used was Salmonella typhi Ty21a (Chapter 2, section V,2,iii). Each vaccine dose contained 10^{11} freshly harvested live organisms suspended in 10 ml of 0.9% sodium chloride.

c) Study Design

Vaccination procedure - after an 8 hour fast all the subjects were given 50ml of 2% sodium bicarbonate solution. The vaccine was administered 5 minutes later in 100 ml distilled water. Three doses each containing $1x10^{11}$ organisms were administered 48 hours apart.

Samples of blood were obtained before vaccination and on days 7, 10, 14 and 21 after vaccination.

Serum was stored in aliquots at -20°C until the assays were performed.

d. Assays

Serum pIgA antibody to typhoid LPS was measured by RIA as described in Chapter 6. Total serum pIgA was measured as described in Chapter 5, section II. Total serum IgA antibody (monomeric and pIgA) IgG and IgM antibody to typhoid LPS was measured by ELISA (Chapter 2, section V,3). Total serum IgA was measured by single radial immunodiffusion (Chapter 2, section V,4).

3. Results

The vaccine was well-tolerated with no adverse effects reported. None of the patients had serum pIgA antibody to typhoid LPS detectable before vaccination. One of the 5 patients developed pIgA antibody to typhoid after vaccination; 18 units per 100ul on day 7 and 25 units per 100ul on day 10 after vaccination. This pIgA antibody could not be detected 16 days after vaccination.

Total serum pIgA levels ranged from 26 ug per ml to 1250 ug per ml (mean 282 ug per ml, median 42.5 ug per ml) (see Table 8.1). The patient with the highest level of serum pIgA was the one in whom antityphoid

pIgA antibody was detected after vaccination. Normal controls have total pIgA levels less than 120 ug per ml (Chapters 6 and 7). Only one of the 5 patients had a higher concentration of total serum pIgA.

There was a mean 4 fold rise in serum IgA antibody to typhoid after vaccination (57.4 ± 53.9 units per 100 ul pre vaccination and 275.2 ± 228.8 units per 100 ul post vaccination; mean ± SD). A greater than 2 fold rise in IgA antibody to typhoid occurred in 3 of the 5 patients (Table 8.2). The peak serum IgA antityphoid level occurred on day 7 or 10 after vaccination. An IgG and IgM class antityphoid antibody response was detected in some of the subjects (Table 8.3).

The total serum IgA levels (Table 8.1) ranged from 1580 ug per ml to 4400 ug per ml (mean 2864 ug per ml). The patient with the highest level of serum pIgA (1250 ug per ml) had 2050 ug per ml of total serum IgA.

TABLE 8.1

Total serum pIgA (RIA) in ug per ml and total serum IgA (Mancini) ug per ml

Subject	pIgA	Total IgA
1	26	2090
2	1250*	2050
3	27	1580
4	68	4400
5	42.5	4200

* Subject 2 the only one to have pIgA to typhoid after vaccination.

TABLE 8.2

Serum IgA antibody to typhoid LPS (ELISA) in units per 100 ul

Subject	Before Vaccination	After Vaccination	Fold Rise
1	25	36 [1]	-
2	30	300 [2]	10
3	22	140 [2]	6.3
4	150	260 [1]	1.7
5	60	640 [1]	10.6
Mean	57.4	275.2	4.7 fold
Range	22-150	36-640	rise

The peak antibody levels after vaccination are shown. These were detected on day 7^{1} or day 10^{2}.

TABLE 8.3

A. Serum IgG antibody to typhoid LPS in units per 100 ul

Subject	Before Vaccination	After Vaccination	Fold Rise
1	10	53	5
2	24	175	7
3	56	85	1.5
4	25	64	2.5
5	10	60	6
Mean	53	Mean 87.4	Mean
Range	10-56	53-175	1.6 fold

The peak antibody levels (detected 7 days after vaccination) are shown.

B. Serum IgM antibody to typhoid LPS in units per 100 ul

Subject	Before Vaccination	After Vaccination	Fold Rise
1	80	80 [1]	0
2	650	2200 [3]	3.3
3	310	1200 [2]	4
4	100	100 [2]	0
5	100	900 [1]	9
Mean	248	Mean 916	Mean
Range	80-650	80-2200	3.6 fold

The peak antibody levels after vaccination are shown. These were detected on day 7[1], day 10[2] or day 14[3].

4. Discussion

In this small sample of patients with IgA nephropathy an augmented serum IgA antibody response to the oral vaccine Ty21a was not observed. Two of the 5 patients did not have a rise in serum IgA antibody to typhoid LPS after vaccination. Only one of the 5 patients developed serum pIgA antibody to typhoid LPS after oral vaccination. This same patient had the highest level of total serum pIgA well above the range of levels measured in normal subjects using the SC binding assay. This patient with elevated serum pIgA did not have elevated total serum IgA. In fact the level of total serum IgA was elevated in only 2 members of the group. The total serum pIgA measured in 4 of the 5 patients was well within the range of total pIgA measured in normal subjects (i.e. <120 ug per ml; Chapter 6 and 7).

Earlier studies have suggested that the parenteral administration of antigens including influenza virus haemagglutinins and inactivated mumps virus (Endoh et al, 1984; Pasternack et al, 1986) results in an augmented IgA response in these patients with IgA nephropathy. Leinniki et al (1987) reported an enhanced IgA antibody response to the oral polio vaccine in 51 patients with IgA nephropathy compared with normal controls. However, not all the patients were high responders in the study of Leinniki. Similar findings were made by Endoh et al (1984). Leinikki

et al, suggested that the triggering antigen for an over-reaction in the IgA antibody response may be individually variable. In this small study with an oral typhoid vaccine, an augmented IgA response was not seen. This may reflect the small number of patients selected or the antigen used.

Only one of the 5 patients in this study had an elevated serum pIgA concentration. Not all patients with the disease have elevated pIgA levels in serum. For instance 4 of the 15 patients studied by Lopez Trascasa et al (1980) did not have elevated serum pIgA. They suggested that the polymerization of IgA may be transient. Delacroix et al (1983) reported that the level of total serum IgA was elevated in a group of 11 unselected patients with IgA nephropathy and that the proportion of serum pIgA with respect to the level of total serum IgA was not elevated in these patients. Newkirk et al (1983) also felt that the elevated pIgA levels found in patients with IgA nephropathy appeared to be a consequence of a general increase in serum IgA. When expressed as a percentage of the total serum IgA no selective elevation of the polymer fraction was observed. In the study reported by Newkirk et al sera from 30 patients were analysed. 38% of the group had elevated total serum IgA and 47% had elevated pIgA, a significant elevation in both total sera IgA and pIgA when compared to normal individuals. 19 of the patients with

IgA nephropathy had total serum IgA levels of 1.9 ± 0.11 mg/ml. The 11 patients with elevated IgA were estimated to have a mean serum IgA level of 3.82 ± 0.24 mg/ml. Therefore only 2 of the 5 patients in the present study could be considered to have elevated IgA levels. Neither of them had high serum pIgA. The one subject with high pIgA comprising 50% of the total serum IgA had a relatively normal total serum IgA level (2050 ug per ml).

The lack of elevated levels of IgA and pIgA and the lack of a heightened immune response to the typhoid vaccine suggests that some of the reported abnormalities in serum IgA and immune reactivity may be transient and dependent on the stage of the disease. In this small sample of patients with stable disease no consistent elevation in pIgA or evidence of heightened immune reactivity to oral vaccination was detected. Ideally, an assessment of the local intestinal immune response to the vaccine would have been desirable. Unfortunately, none of the pateints were prepared to undergo intestinal intubation.

In summary, in this small study elevated levels of serum pIgA were not found in all the patients with IgA nephropathy and the immune respone to the oral typhoid vaccine was not augmented when compared to the immune response to the vaccine in normal subjects.

IV. SERUM POLYMERIC IgA AND ANTIBODY TO ESCHERICHIA COLI LIPOPOLYSACCHARIDE IN PATIENTS WITH CROHN'S DISEASE

1. Introduction

Crohn's disease (regional enteritis) and ulcerative colitis are chronic inflammatory disorders of the intestine of unknown aetiology. In these disorders the intestinal mucosa is infiltrated with lymphocytes and plasma cells. These factors have led to the suggestion that inflammatory bowel disease may be caused or perpetuated by immune mechanisms (Kraft and Kirsner, 1971).

One of the features of Crohn's disease is a pronounced local humoral immune response with an increase in the number of mucosal IgA, IgM and IgG immunocytes (Baklien and Brandtzaeg, 1975). In addition to the reported 2-fold increase in the number of IgA immunocytes there are alterations in local IgA production. There is a shift towards local IgA1 production (MacDermott et al, 1986; Kett and Brandtzaeg, 1987) and a shift towards the local production of monomeric IgA (Brandtzaeg and Korsrud, 1984; MacDermott et al, 1986). The possibility that these changes were due to IgA1 secreting cells and monomeric IgA producing cells being attracted to the inflamed mucosa was suggested by MacDermott et al (1986).

In peripheral blood, mononuclear cells from patients with Crohn's disease have shown increased spontaneous IgA secretion when compared to the peripheral blood mononuclear cells of normal controls (MacDermott et al, 1981; MacDermott et al, 1983). In addition to these findings an increase in the polymeric fraction of serum IgA and an increase in the proportion of IgA2 in serum has been reported in these patients (Delacroix et al, 1983).

As there is increased production of immunoglobulin within the mucosa the possibility of immune stimulation by bacteria has led investigators to measure serum antibody to numerous bacteria in Crohn's disease (Matthews et al, 1980). Increased levels of serum antibody to many bacteria have been reported (Brown and Lee, 1974; Blaser et al, 1984) and serum antibody to many strains of Escherichia coli (E.coli) appear to be increased in patients with Crohn's disease (Tabaqchali et al, 1978) and ulcerative colitis (Tabaqchali et al, 1978; Heddle and Shearman, 1979). These studies measured agglutinating antibodies against E.coli antigen with therefore an inherent bias towards the detection of IgM antibody.

In the study reported here the SC binding assays were used to measure the total serum pIgA and the serum pIgA antibody to E.coli LPS in a group of patients with Crohn's disease. The total serum IgA antibody to E.coli LPS and

the IgG and IgM antibody to E.coli LPS were measured by enzyme linked immunosorbent assays (ELISA).

2. Materials And Methods

a) Subjects

Sera were obtained from 26 patients with Crohn's Disease and 13 healthy controls. The patients (median age 25 years; range 18-64 years) had clinical, radiological endoscopic and biopsy findings consistent with Crohn's disease. Disease activity was determined using the Harvey Bradshaw Index (Harvey and Bradshaw, 1980). Disease activity was moderate to severe.

b) Assays

E.coli LPS specific pIgA antibody was measured as described in chapter 3, section II. The ELISA for E.coli LPS-specific IgA, IgG and IgM antibody was performed as described in chapter 2, section V,3.

Total serum pIgA was measured by RIA (Chapter 5, section II).

Total immunoglobulin in the IgA, IgG and IgM class were measured by single radial immunodiffusion (Chapter 2, section V,4).

3. Results

Immunoglobulin levels

As a group the patients with Crohn's disease did not have elevated levels of serum pIgA (Figure 8.4). The concentrations of pIgA in patients sera and control sera were 67±106 and 39±26ug per ml (mean ± SD) respectively.

Three of the 26 patients did have elevated levels of serum pIgA. These 3 patients had total IgA levels within the normal range. Although all 3 of these patients had moderate disease activity (Harvey Bradshaw index score 5to10) two went to surgery for long standing ileitis. The third had an ileostomy.

Total serum IgA levels in the 26 patients were not elevated when compared to the total serum IgA levels in the controls (Figure 8.5). The mean IgA levels in the patients and controls were 1729±620 and 1329±777 ug per ml respectively. Similarly the IgG and IgM levels were not elevated (IgG level 7015±2531ug per ml in patients; 6780±1367ug per ml in controls; IgM level 1869±770ug per ml in patients and 1696±709ug per ml in controls).

Antibody to **E.coli** LPS

Antibody to E.coli LPS was detected in both patient and control sera (Figure 8.6). There was no significant difference between the level of antibody to E.coli LPS

between patient and control sera with respect to any of the 3 immunoglobulin classes. The antibody level to E.coli LPS in units per 100ul (mean ± SD) in patients and normal controls were 538±646 and 361±388 for IgA antibody, 286±361 and 257±323 for IgG antibody and 2061±1430 and 1910±1357 for IgM antibody respectively.

pIgA antibody to E.coli LPS was detected in 6 of the 26 patients with Crohn's disease (Table 8.4). All 6 had moderate disease activity (score 5 to 10; Harvey Bradshaw Index). One of the 6 had colitis. The other 5 (one of whom had an ileostomy) had ileitis. One of the 6 had a high concentration of total serum pIgA (Table 8.4) and 3 of the 6 had high total IgA antibody to E.coli LPS. However, the patient with the highest level of IgA antibody to E.coli LPS did not have any pIgA E.coli antibody.

TABLE 8.4

PATIENT	pIgA to E.coli LPS UNITS per 100ul	Total IgA to E.coli UNITS per 100ul	TOTAL pIgA ug/ml	TOTAL IgA ug/ml
1	140	360	67	1690
2	82	2000	83	2250
3	34	100	560	1310
4	32	1700	63	1500
5	32	1000	35	1530
6	22	300	40	2630

4. Discussion

In this study only 3 of the 26 patients with Crohn's disease had elevated levels of total serum pIgA. This contrasts with the report of Delacroix et al (1983), who reported a preferential increase in pIgA in a group of 12 patients with Crohn's disease. However, the patients in their study were a select group with total serum IgA levels >2 standard deviation above the IgA levels of the normal controls. The patients in the study reported here did not have elevated IgA levels. The reason why 3 of them had elevated pIgA is unclear. These 3 patients did not have elevated total IgA levels.

There is conflicting data with regard to serum IgA levels in Crohn's disease. Several workers have reported an increase in serum IgA (Hobbs and Hepner, 1968; Deodhar, Michener and Farmer, 1969; Bolton et al, 1974), but this has not been a consistent finding (Bendixen et al, 1968; Weeke and Jarnum, 1971; Bergman et al, 1973) and recent reviews on the immunology of Crohn's disease suggest that the IgA levels are usually normal (Kirsner and Shorter, 1982; Jewell and Patel, 1985). The patients in the present study had normal serum IgA levels.

At the level of the local immune system in Crohn's disease there is an increase in the number of IgA cells in the mucosa (Baklien and Brandtzaeg, 1975). IgA cells from

the intestine preferentially produce pIgA containing J chain (Brandtzaeg, 1973; Kutteh et al, 1982a) whereas IgA cells from the bone marrow preferentially produce monomeric IgA (Radl et al, 1974). An increase in the mucosal IgA cells might have been expected to lead to a increase in serum pIgA. However, a switch toward the production of monomeric IgA by the IgA cells in the decreased mucosa has been reported (MacDermott et al, 1986; Kett and Brandtzaeg, 1987). This might explain the normal pIgA levels in the patients in this study.

Little is known about the antibody specificities of the local immunoglobulin producing cells in Crohn's disease. Heddle et al (1982) found that intestinal mononuclear cells from patients with Crohn's disease produced antibodies to common faecal E.coli strains in vitro. Increased levels of serum antibody to many bacteria have been reported in Crohn's disease (Matthews et al, 1980; Blaser et al, 1984). It has been suggested that the gut wall of these patients is more permeable to luminal bacterial macromolecules which may stimulate the gut associated lymphoid tissue (Shorter et al, 1972).

However, the data is conflicting. Tabaqchali et al (1978) found increased serum agglutinin to a variety of E.coli serotypes in patients with Crohn's disease. On the other hand Brown and Lee (1973) did not find elevated

serum antibody to E.coli. Kruis et al (1984) reported increased serum antibody to Lipid A, a component common to the endotoxin of gram negative bacteria.

However, Nolan et al (1986) and Kobayashi et al (1988) did not find abnormal levels of antibody to Lipid A in Crohn's disease patients.

In the previous study patients with alcoholic liver disease had significantly higher levels of serum IgA than normal controls and significantly higher levels of IgA antibody to E.coli LPS (Chapter 7). The patients with alcoholic liver disease had elevated total serum pIgA and serum pIgA antibody to E.coli LPS. In the present study in Crohn's disease using the same E.coli LPS preparation that was utilized in the previous study only 6 of the Crohn's disease patients had serum pIgA to E.coli LPS and the levels of IgA antibody to E.coli LPS were not significantly higher than the level in controls. This suggests that decreased hepatic clearance of antigens or decreased clearance of pIgA rather than damage to the intestinal epithelium was reponsible for the findings in the liver disease patients.

In summary no increase in pIgA levels, total IgA levels or antibody to E.coli LPS was detected in this group of patients with Crohn's disease.

V. SERUM POLYMERIC IgA ANTIBODY TO CAMPYLOBACTER JEJUNI IN PATIENTS WITH CAMPYLOBACTER ENTERITIS

1. Introduction

Campylobacter jejuni is the most common bacterial cause of diarrhoea in developed countries (Blaser and Reller, 1981). The pathogenesis of the human infection is not completely understood but the clinical characteristics of bloody diarrhoea, cellular infiltration of the lamina propria, fever and bacteraemia suggest that they are invasive organisms (Blaser and Reller, 1981). Patients with Campylobacter enteritis mount both an intestinal and systemic immune response to the infection. Winsor et al (1986) using western blot analysis demonstrated the presence of secretory IgA antibody to Campylobacter outer membrane in faecal extracts from patients with Campylobacter enteritis. A transient IgA response in stool extracts was detected by Lane et al (1987) using an enzyme linked immunosorbent assay.

A serum antibody response has also been demonstrated (Kaldor et al, 1983; Blaser and Duncan, 1984). Blaser and Duncan used an ELISA to measure serum antibody and were able to demonstrate rising serum IgA, IgG and IgM antibody during the second week of infection.

In this study the serum antibody response to

Campylobacter jejuni was studied in 13 patients presenting to the Royal Adelaide Hospital with Campylobacter enteritis. An enzyme linked immunosorbent assay was used to detect antibody in the IgA, IgG and IgM class. The SC binding assays were used to measure serum pIgA antibody to Campylobacter and total serum pIgA.

2. Materials And Methods

a) Subjects

Samples of serum from 13 patients presenting to the Royal Adelaide Hospital with Campylobacter enteritis and 8 healthy laboratory staff were studied. Serum was obtained 10 days after the illness began. Bacteriological confirmation of the diagnosis was made at the Institute of Medical and Veterinary Science. Samples were stored at $-20^{o}C$ until the assays were performed.

b) Assays

Bacterial Strains And Antigen Preparations

C. jejuni strains PEN1, PEN2 and PEN3 were used (Chapter 2, section 5,3,iv).

Equal amounts of each of the 3 antigen preparations were diluted in 0.5 M Sodium Carbonate buffer pH9.6 and combined to yield a total protein content of 5 ug per ml.

Microtitre plates with 96 wells (COSTAR Data Packaging Corp, Cambridge, Mass. USA) were used for the ELISAs and RIAs. 100 ul of the antigens in Sodium Carbonate buffer (5 ug/ml) were added to each well. After overnight incubation at 4°C the wells were emptied, blocked with 150 ul of 0.5% BSA in PBS for 45 minutes at room temperature and then washed with PBS Tween 20.

Radioimmunoassay For pIgA Antibody To Campylobacter

The radioimmunoassay for pIgA antibody to Campylobacter antigens was performed as described in Chapter 3, section II.

ELISA For IgA, IgG And IgM Antibody To Campylobacter

The ELISA was performed as described in Chapter 2, section V,3,b).

Radioimmunoassay For Total Serum pIgA

Total serum pIgA was measured as described in Chapter 5, section II.

Immunodiffusion Assays

Total serum immunoglobulin in the IgA (monomeric and pIgA), IgG and IgM class was measured by single radial immunodiffusion (Chapter 2, section V,4).

3. Results

8 of the 13 patients had serum pIgA antibody to Campylobacter (mean ± SD = 105 ± 170 units per 100 ul). None of the controls had serum pIgA to Campylobacter (see Figure 8.7).

The ELISA detected IgA antibody (i.e. both monomeric and pIgA antibody) to Campylobacter in both patients and controls (Figure 8.8); 3922±4562 and 218±250 antibody units per 100 ul respectively. This difference was significant (u=6, p=.002). IgG and IgM antibody was detected in sera from both patients and controls (Figure 8.8). IgG antibody to Campylobacter 7513±6959 units per 100 ul for patients and 4161±5168 units per 100 ul for controls. This difference was not significant at the .10 level of significance. The levels of IgM antibody between patients and controls were also not significantly different 2330±2694 and 876±794 units per 100 ul for patients and controls respectively.

Only one patient had an elevated concentration of total serum pIgA (450 ug per ml). The other patients had levels less than 120 ug per ml, the upper limit of total pIgA in normal sera (Chapters 6 and 7). The mean (±SD) total pIgA levels in patients was 76±116 ug per ml and in the 8 controls was 25±14 ug per ml. The total IgA levels were 1667±842 and 1940±383 ug per ml for patients and

controls respectively. The total IgG concentration was 7673±2112 ug per ml for patients and 10,240±1881 ug per ml for controls. The IgM levels were 1200±513 ug per ml and 1501±444 ug per ml for patients and controls respectively.

There was a significant correlation between the serum pIgA antibody to Campylobacter and the total IgA antibody to Campylobacter (r_s=0.764; critical value of r_s at the 0.05 level of significance for the one tailed test = 0.506).

4. Discussion

In this study an early serum IgA antibody response to Campylobacter enteritis was detected. The patients had significantly higher levels of IgA antibody to Campylobacter than the controls. pIgA antibody to Campylobacter was only detected in patients. There was a correlation between the level of serum pIgA antibody and the total serum IgA antibody to Campylobacter.

Blaser and Duncan (1984) reported that the IgA, IgG and IgM antibody to Campylobacter was significantly raised in the second week after infection and that the IgG and IgM antibody elevation persisted for longer (30 to 40 days) than the IgA antibody rise which was early and transient. They felt that the IgA ELISA had the better discriminating power between patients and controls.

Campylobacter jejuni causes tissue injury to the jejunum, ileum and colon. A bloody, edematous and exudative enteritis has been observed (Blaser and Reller, 1981). In some intestinal disorders characterised by mucosal inflammation elevated levels of pIgA have been found. An elevation in the serum pIgA level was reported in Crohn's disease (Delacroix et al, 1983). A previous study (Section II) also revealed increased levels of total pIgA in the patients with coeliac disease. However, there was no elevation in total serum pIgA in the patients with Campylobacter enteritis, perhaps reflecting the transient nature of the inflammation.

The results of this study are in keeping with those found in the study where the serum pIgA antibody response to oral typhoid vaccination was examined, though limited by lack of information on the temporal profile of the pIgA response and data on intestinal antibody.

It is difficult to speculate on the origin of the serum pIgA antibody to Campylobacter in these patients. They may be gut derived spilling out into the circulation. However, C. jejuni is invasive and transient bacteraemia is often observed (Blaser and Reller, 1981). Therefore the pIgA antibody may equally well have been produced in the lymphoid tissues of the vascular rather than the mucosal

compartment. The correlation between the pIgA antibody to Campylobacter and the total serum IgA to Campylobacter tends to support this.

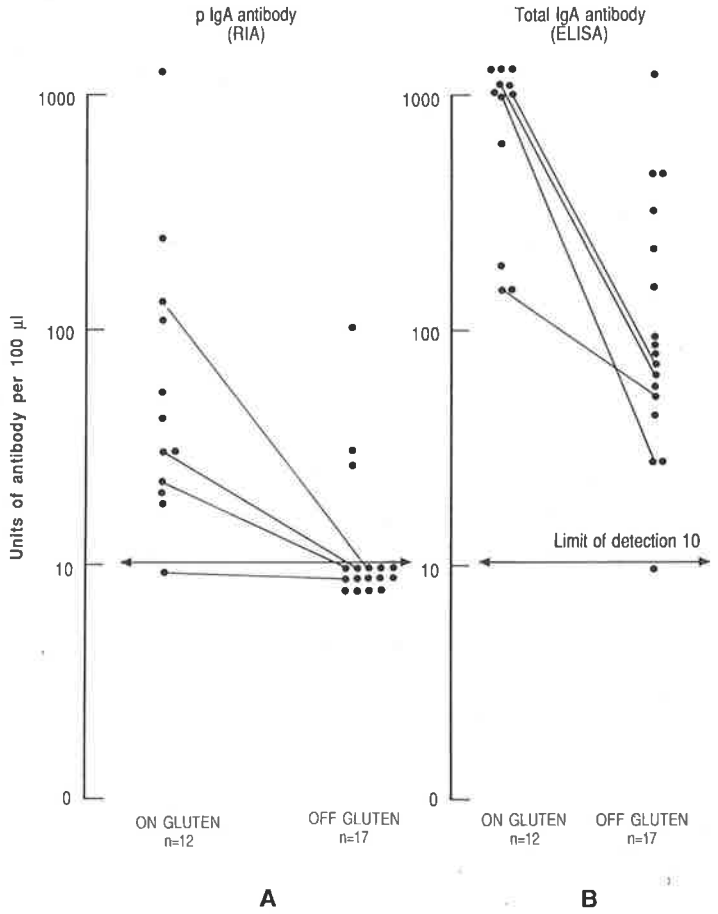


Figure 8.1

Antibody to gliadin in subjects from whom only serum samples were obtained in units of antibody per 100ul.

A. Serum pIgA antibody to gliadin in subjects on gluten (n=12) and off gluten (n=17).

B. Total serum IgA antibody to gliadin (monomeric plus pIgA) in subjects on gluten (n=12) and off gluten (n=17).

The lines indicate paired samples from the same subjects (n=4).

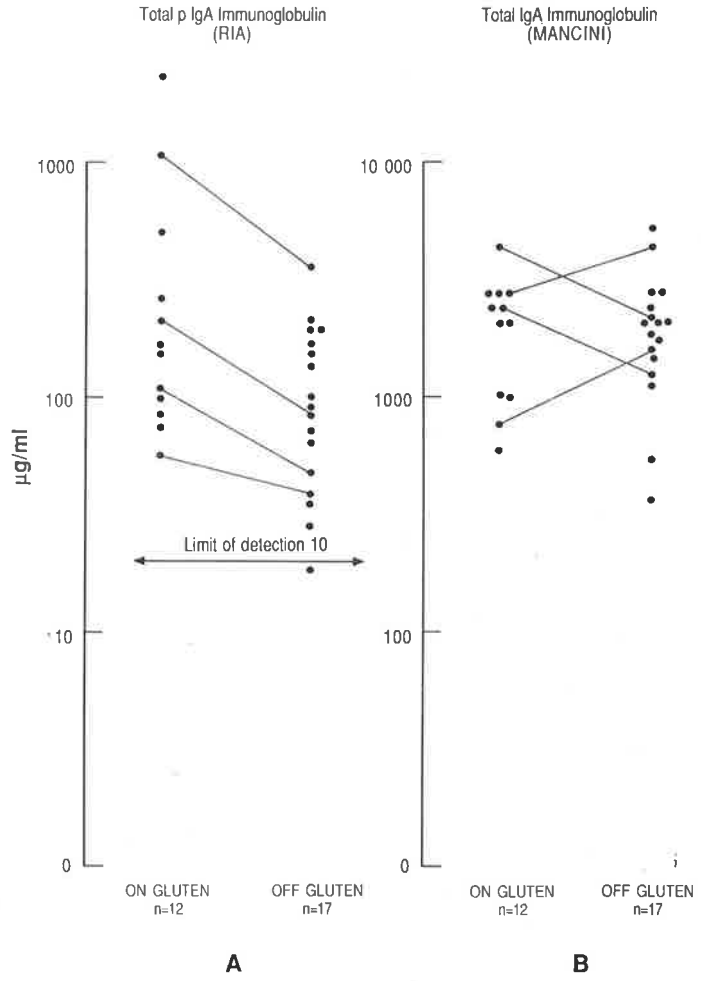


Figure 8.2

Total serum IgA immunoglobulin levels in subjects from whom only serum was obtained while on gluten (n=12) or off gluten (n=17).

A. Serum pIgA measured by RIA.

B. Total serum IgA (monomeric plus pIgA) measured by single radial immunodiffusion.

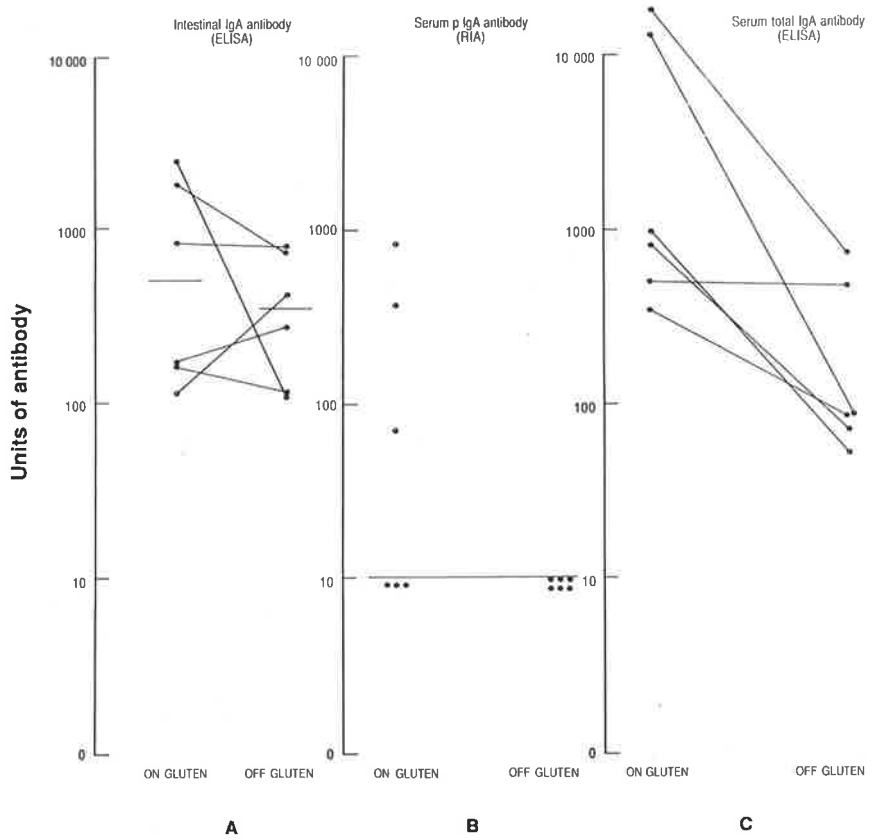


Figure 8.3

Anti gliadin antibody in children in whom paired samples of both serum and intestinal fluid were obtained when they were on and off gluten.

A. Intestinal IgA antibody to gliadin measured by ELISA.

B. Serum pIgA antibody to gliadin measured by RIA.

C. Serum total IgA antibody to gliadin measured by ELISA.

Results are expressed as units of antibody per mg of immunoglobulin for intestinal fluid and as units of antibody per 100ul for serum

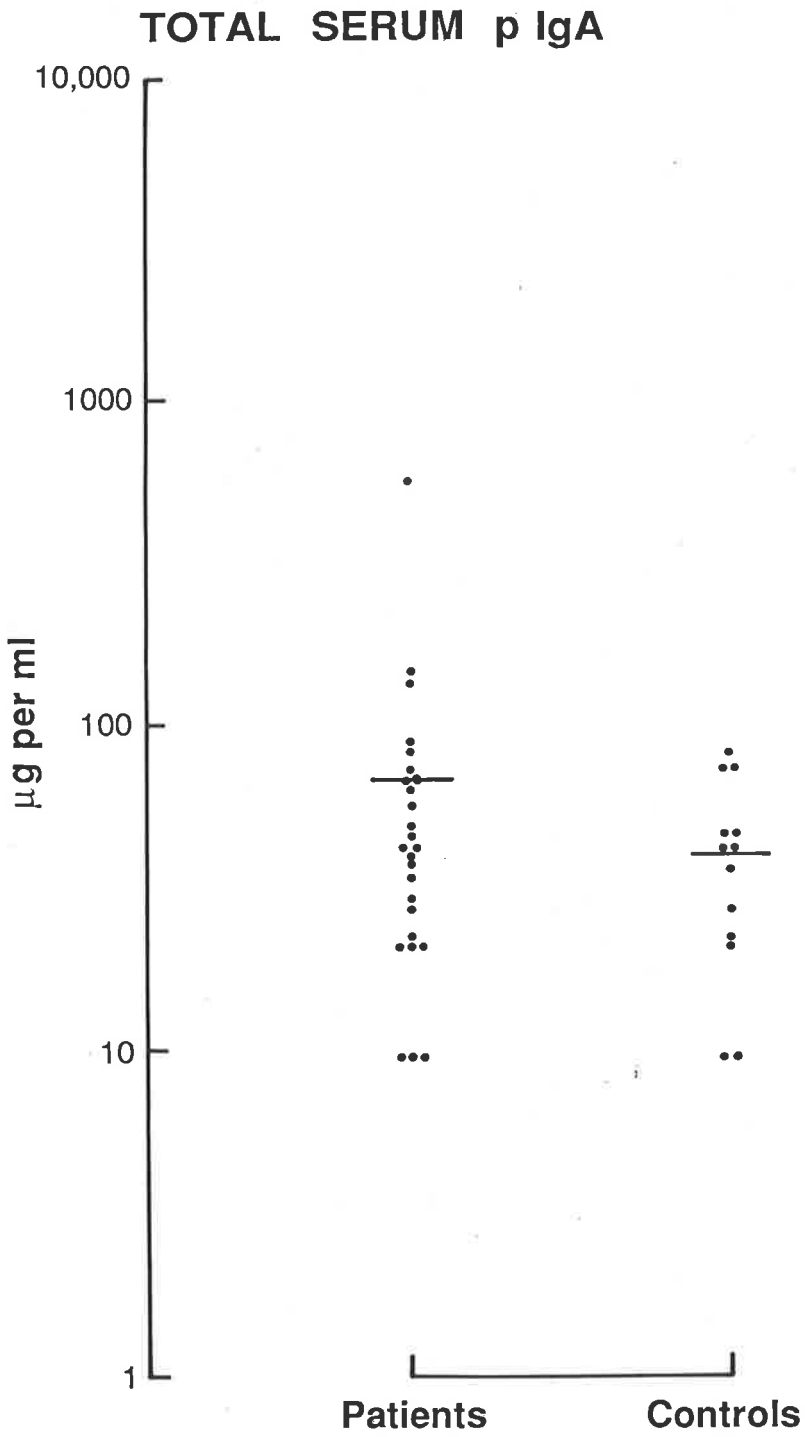


Figure 8.4

Total serum pIgA in patients with Crohn's disease (n=26) and control subjects (n=13). Horizontal bars indicate means.

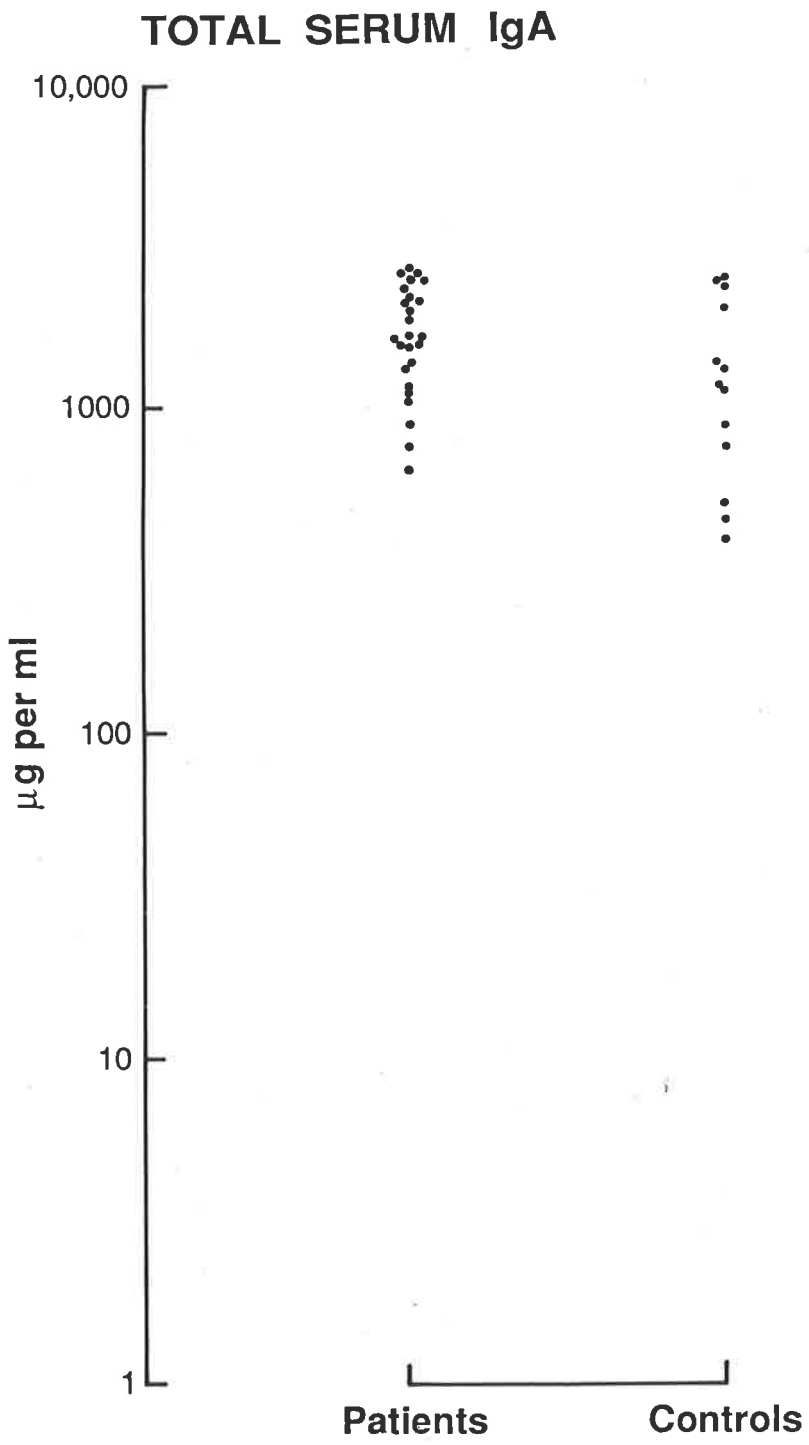


Figure 8.5

Total serum IgA levels (monomeric plus pIgA) in patients with Crohn's disease (n=26) and control subjects (n=13). Total IgA levels were measured by single radial immunodiffusion.

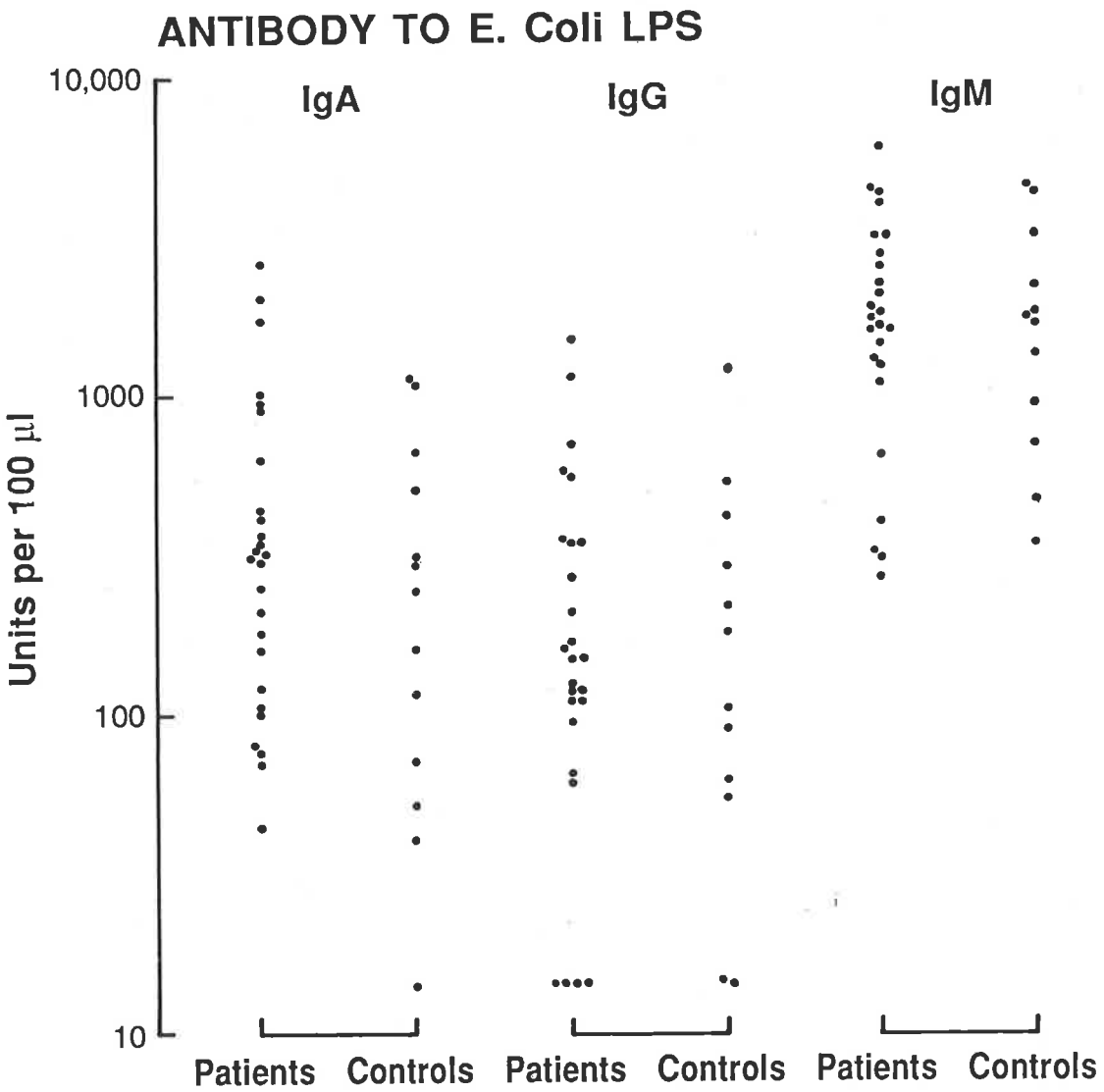


Figure 8.6

Serum antibody to E.coli LPS in patients with Crohn's disease (n=26) and healthy controls (n=13). IgA antibody (monomeric plus pIgA), IgG antibody, IgM antibody; from left to right respectively.

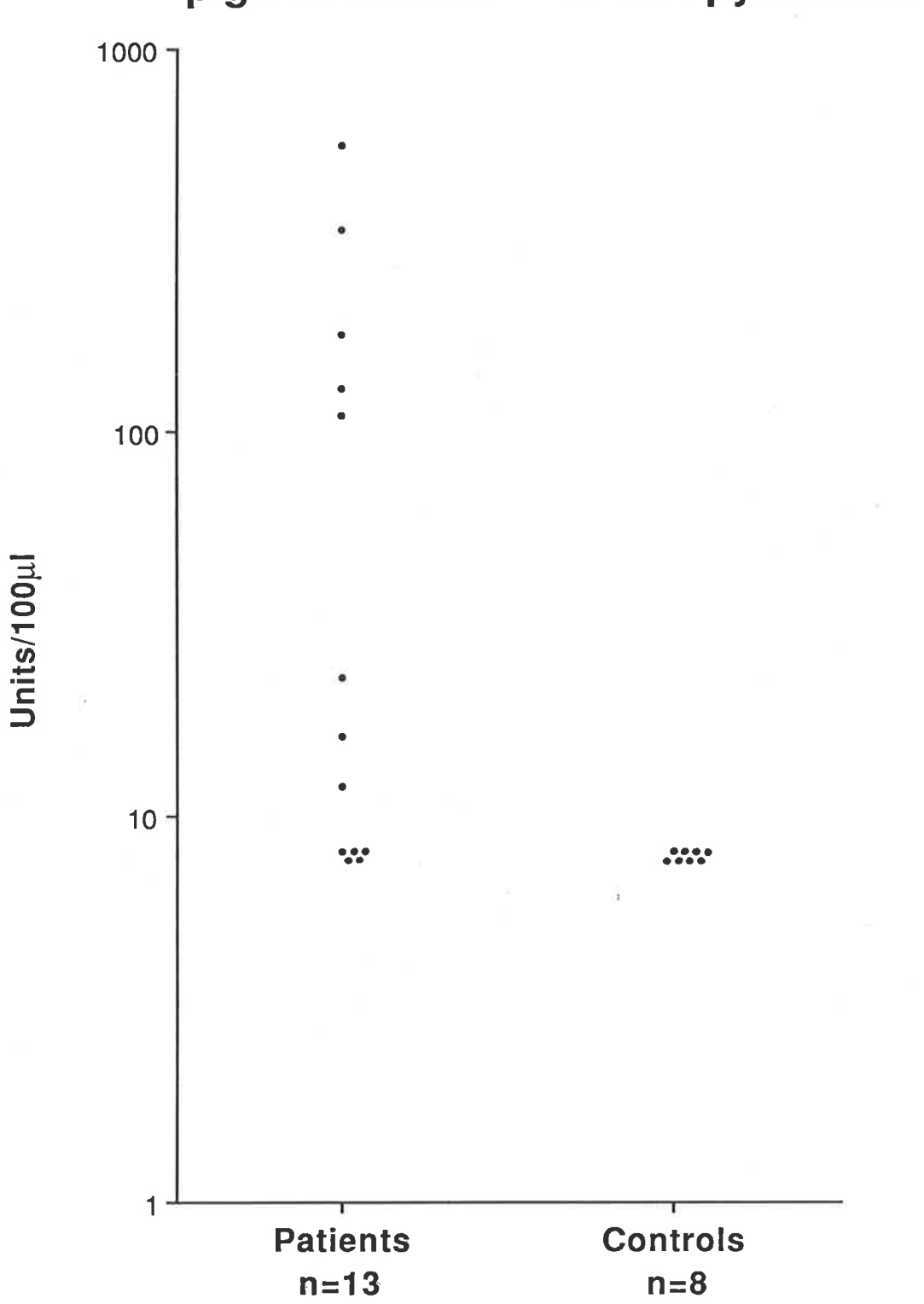


Figure 8.7

pIgA antibody to Campylobacter antigens in patients with Campylobacter enteritis (n=13) and healthy controls (n=8). Limit of detection 10U of antibody per 100ul.

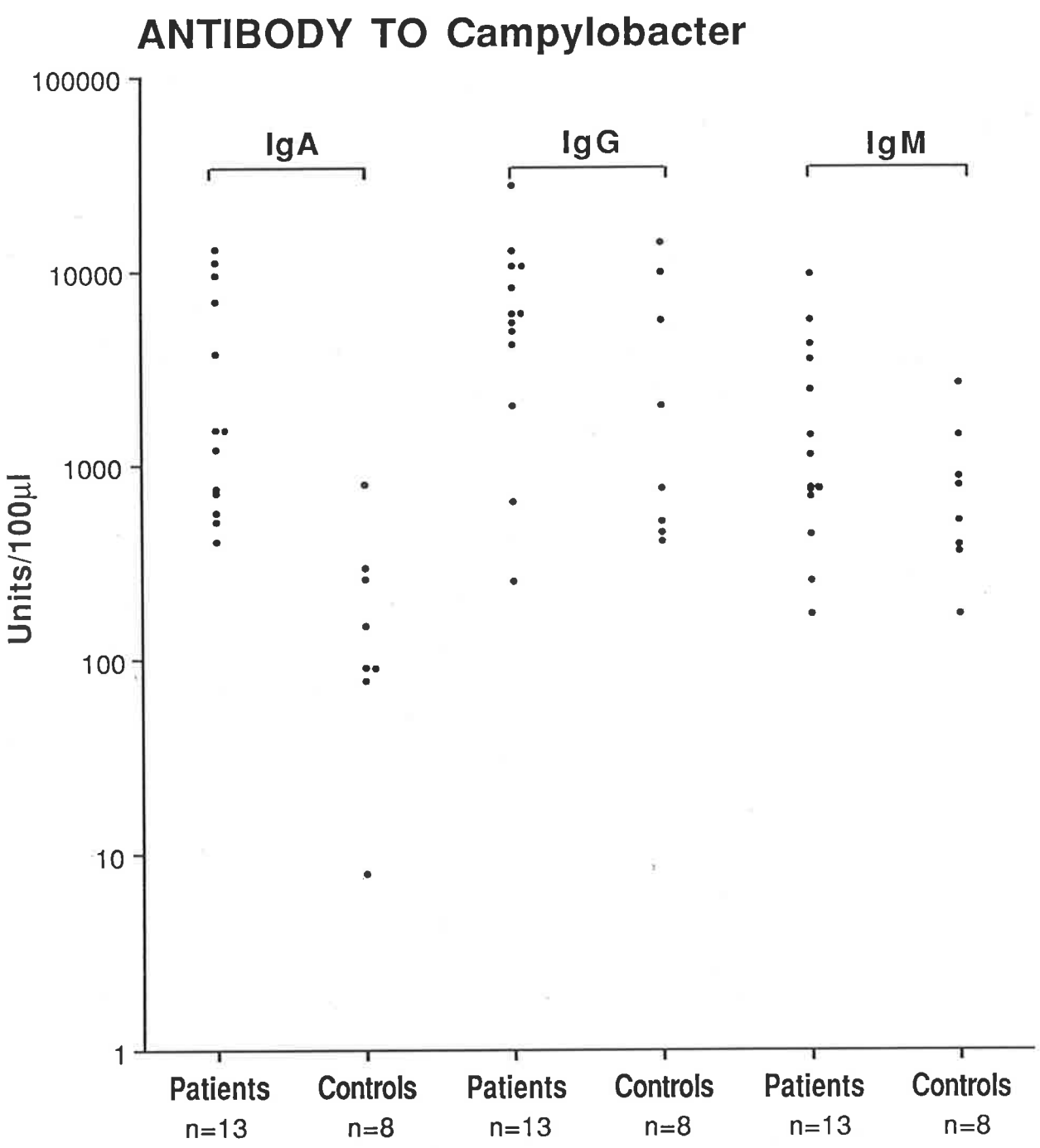


Figure 8.8

Antibody to Campylobacter antigens in patients with Campylobacter enteritis (n=13) and control subjects (n=8). Total IgA antibody (monomeric plus pIgA), IgG antibody and IgM antibody from left to right.

CHAPTER 9 - DISCUSSION

Ever since Besredka proposed that a local immune system existed in the intestine functioning independently of the systemic immune system (Besredka, 1919) and the importance of secretory IgA in mucosal secretions was recognised, there have been numerous attempts to measure intestinal immune responses by measuring antibody levels in intestinal secretions. The potential role of immunological factors in some intestinal diseases and the concept of generating local immunity against enteric diseases by oral immunisation has given added impetus to studies of the intestinal immune response.

Intestinal antibody formation in humans has been monitored by measuring antibody levels in faeces, duodenal aspirates and intestinal lavage specimens (reviewed in Chapter 1, section IV). Each of these procedures has limitations. Immunoglobulins in faecal specimens are often extensively degraded by intestinal and bacterial enzymes and the collection of duodenal or lavage specimens is a laborious process (see Chapter 1, section IV). There exists a need for simple methods of assessing intestinal immune responses in humans, particularly methods that could be utilised in studies of large populations for example in relation to field trials of oral vaccines.

In view of the concept of a common mucosal immune system (Bienenstock, 1974), various methods of indirectly examining the intestinal immune response have been explored (Reviewed in Chapter 1; Section IV,2). The use of other body fluids such as serum and saliva for assessing intestinal immunity indirectly has not been a universal success. Despite the claims made by some investigators the measurement of even serum IgA antibody has been of limited value as they do not necessarily reflect the intestinal immune response (LaBrooy et al, 1980; Chau et al, 1981; Jertborn et al, 1986).

The hypothesis that was put to the test in the present study was that the measurement of antibody in the polymeric fraction of serum IgA in humans would provide a reliable correlate of the secretory IgA antibody response in the intestine and thereby provide a more readily available measure of the immune response in the intestine.

The reason for focussing on the small fraction of IgA in serum that is polymeric was the suggestion that it could be derived from intestinal IgA cells (Heremans, 1974a; Lamm, 1976) in contrast to the vast bulk of serum IgA in humans which is monomeric and derived from IgA cells in the bone marrow (Hijmans et al, 1971).

In order to test the hypothesis two requirements had to be met. Firstly, an assay of suitable sensitivity and specificity capable of measuring antigen specific pIgA in serum was needed. Secondly, a well defined situation where an antibody response to an antigenic stimulus delivered to the secretory immune system could be expected.

No easy method of measuring antigen specific pIgA was available when the present study was begun. Serum IgA had been separated into polymeric and monomeric fractions by velocity sedimentation in sucrose density gradients or by gel chromatography (Lopez Trascasa et al, 1980; Sancho et al, 1982; Kutteh et al, 1982a&b; Delacroix et al, 1983) and a SC binding assay for total serum pIgA had been described (Newkirk et al, 1983). One possible method of measuring antigen specific pIgA was to perform immunoassays on fractions obtained after gel chromatography or sucrose density gradient ultra centrifugation. It was felt that this would be time consuming and could result in loss of material during fractionation. However, Russel et al (1986) have used solid phase radioimmunoassays performed on polymeric and monomeric fractions of serum IgA separated by high performance, size exclusion chromatography (HPLC) to determine the molecular forms of IgA antibody to a variety of food, bacterial and viral antigens.

In view of the affinity of SC for pIgA (Hanson et al, 1969; Mach, 1970; Radl et al, 1971; Brandtzaeg, 1971; Lindh and Björk, 1974; Weicker and Underdown, 1975) the use of labelled SC to detect solid phase antigen specific pIgA in serum which bound to antigen coated micro titre plates was potentially a simpler method of measuring pIgA antibody in multiple samples.

Evidence that SC would bind to liquid phase pIgA *in vitro* already existed (see above) and Newkirk et al (1983) had described an assay for quantitating the total pIgA in serum using anti-IgA antibodies to precipitate ^{125}I labelled SC coupled to pIgA.

In this thesis the development of a new solid phase assay for measuring antigen specific pIgA in human serum has been described. The assay is simple in concept and can be performed relatively rapidly.

Since SC binds avidly to IgM (Brandtzaeg, 1977; Socken and Underdown, 1978) the detection of pIgA antibodies necessitated the removal of IgM from serum samples. This was achieved through the use of small immunoadsorbent columns containing IgM specific antibodies which completely depleted 0.5ml aliquots of serum of IgM yielding samples in which the antibody detected with SC

was wholly pIgA. This approach to remove IgM was chosen in order to avoid the possibility of denaturing the pIgA as could occur in attempting to isolate IgA by immunoadsorption. In all the assays performed the removal of IgM was confirmed by ELISA and radial immunodiffusion. However, the 5-10 fold dilutions resulting from the additional step may prevent the detection of low levels of pIgA antibody in serum.

In addition to an assay for antigen specific pIgA, an assay for measuring the total pIgA in serum based on the inhibition of binding of SC to polymeric immunoglobulins (IgM) by pIgA in aliquots of IgM depleted serum has been described. This assay was developed as the quantity of antigen specific pIgA antibody may have been influenced by the total pIgA in serum and levels of pIgA had been reported to be elevated in diseases such as liver disease (Tomasi and Grey, 1972; André and André, 1976; Kutteh et al, 1982b; Delacroix et al, 1983), IgA nephropathy (Lopez Trascasa et al, 1980) and Crohn's disease (Delacroix et al, 1983), diseases in which antigen specific pIgA antibody was examined in the studies reported here. In this assay for total pIgA, one preparation of monoclonal IgM was used to coat the plates in view of the minor differences in the binding of labelled SC to the preparations of purified monoclonal and polyclonal pIgA and IgM tested during development of the assay. One

preparation of polyclonal pIgA was used as the standard in all the assays for total pIgA. The assay was used to estimate total pIgA in serum of normal subjects (Chapter 6) as well as patients with alcoholic liver disease (Chapter 7), coeliac disease and IgA nephropathy (Chapter 8).

There were problems associated with the use of this radioimmunoassay. In addition to the problems inherent to most radioimmunoassays which are decay of the radiolabel and stability of the radiolabelled protein the incorporation of ^{125}I into SC had an adverse effect on the binding of SC to pIgA. The binding of ^{125}I labelled SC to solid phase pIgA was less than anticipated (Chapter 3).

Therefore attempts were made to substitute the radioimmunoassay with an enzyme based ELISA. Two strategies were evaluated. Firstly, the use of alkaline phosphatase labelled SC and secondly the use of an alkaline phosphatase labelled anti SC to detect SC bound to pIgA. The first approach failed presumably due to steric hindrance. The alkaline phosphatase labelled anti-SC bound SC avidly and it was used to demonstrate the affinity of SC for solid phase pIgA and confirm the specificity of the binding of SC to pIgA. However, when the ELISA was evaluated as a means of measuring antigen

specific pIgA in serum the extra washing and incubation steps resulted in loss of bound SC, due to dissociation, leading to a lower assay sensitivity which was considered inadequate for the purpose of this study. Despite its limitations the ^{125}I labelled SC binding assay was successfully used to measure antigen specific pIgA antibody in the serum of both normal subjects and patients.

The second requirement for the present study was a defined situation in which the immune response in intestinal fluid and serum could be studied following an antigenic stimulus. The oral typhoid vaccine Ty21a proved to be a safe and useful physiological tool with which to provide a controlled antigenic stimulus to the intestine.

The initial studies confirmed reports of the safety and lack of side effects of the vaccine and demonstrated that an intestinal antibody response could be reliably generated following the administration of 3 doses of 10^{11} organisms (Chapter 6, section II). This vaccine was then used to assess the relationship between IgA in intestinal fluid and serum pIgA antibody in a number of circumstances.

The studies presented in this thesis clearly demonstrated that a serum pIgA antibody response could be

generated in healthy human volunteers through the delivery of an antigenic stimulus to the intestine. The pIgA response occurred early and was short lived. However, there was no correlation between the levels of serum pIgA antibody to typhoid LPS and intestinal IgA antibody to typhoid LPS after oral vaccination and no evidence of typhoid specific serum pIgA antibody was detected in some of the volunteers who mounted a strong intestinal IgA antibody response. Furthermore delivery of a parenteral antigenic stimulus to normal volunteers, the parenteral heat killed typhoid vaccine, generated typhoid specific pIgA in serum although no intestinal anti-typhoid IgA antibody was produced until these same volunteers were given a course of the oral vaccine.

These studies clearly indicate that there is no direct relationship between intestinal antibody and the generation of pIgA antibody and suggest that although the intestine may be a source of some of the pIgA in serum (as pIgA antibody could be generated by oral immunisation) it was certainly not the only source of pIgA.

Prior to the studies presented in this thesis the relationship between the generation of pIgA antibody in serum and the generation of intestinal IgA following an antigenic stimulus had not been examined in humans. There had been two reports of a serum pIgA response to an

antigenic challenge.

Mascart-Lemone et al (1987) demonstrated a short lived early serum pIgA response to parenteral vaccination with tetanus toxoid. Intestinal IgA antibody was not examined.

Brown et al (1985) reported that experimental infection with Influenza A virus in humans produced a secretory IgA antibody response to the viral hemagglutinin in nasal secretions as well as a predominantly pIgA antibody response in serum. They fractionated serum samples by high pressure liquid chromatography on a size exclusion column and applied these fractions to wells coated with viral hemagglutinin. Brown et al suggested that the serum pIgA to viral hemagglutinin was of mucosal origin but admitted that they could not exclude the possibility that the viral hemagglutinin might have the unique property of stimulating the production of only pIgA even in tissues which are known to produce predominantly monomeric IgA. The time course of the pIgA response was not examined.

Mascart-Lemone et al (1987) suggested that the pIgA antibody to tetanus toxoid was produced in regional lymph nodes draining the site of immunization while pointing out that Kutteh et al (1982a) had demonstrated that 40% of IgA

secreted by cultured lymph node cells was polymeric. The appearance of typhoid specific pIgA antibody in serum despite the absence of intestinal typhoid specific antibody following parenteral typhoid vaccination in the study reported here, also suggests that serum pIgA antibody could originate in extra mucosal sites.

In the study reported by Kutteh et al (1982a), in which human tissues were examined for their ability to produce monomeric and polymeric forms of IgA in vitro intestinal lamina propria cells secreted the greatest amount of pIgA. However, tonsillar, lymph node and peripheral blood cells produced equal proportions of monomeric and pIgA. Peripheral blood lymphocytes stimulated with mitogens produce predominantly pIgA (Kutteh et al, 1980). Although the latter observation was interpreted as evidence for the "maturational" journey of IgA precursor cells from mucosal sites it could also indicate that pIgA could have important functions within the body independent of its function in mucosal secretions. Although the function of the secretory IgA in secretions is well documented (Tomasi, 1976; Lamm, 1976; Underdown and Schiff, 1986) the role of serum IgA remains poorly understood. The function of serum pIgA is even more uncertain. Bienenstock and Befus (1983) postulated that the primary role of pIgA is to clear the circulation of materials that have leaked across the epithelium of

mucosal surfaces. They suggested that the antigen crossing mucosal surfaces could have a high probability of forming complexes with the locally produced specific pIgA antibody. An immunogenic role was also proposed. Bienenstock and Befus (1983) suggest that pIgA complexed to antigen may be selectively transported across hepatic parenchymal cells and into external secretions and that the antigen-antibody complex might provide the mucosal lymphocyte with further antigenic stimulation, regulating local IgA synthesis. In rats pIgA containing immune complexes are removed from the circulation and excreted into bile (Peppard et al, 1981; Russell et al, 1981; Socken et al, 1981). However, in humans the hepatobiliary transport of IgA is less significant (Delacroix et al, 1982c; Dooley et al, 1982) and this method of antigen disposal may not be important.

The factors controlling the production of serum pIgA antibody are not clearly understood. The results of the present study and the work of Mascart Lemone et al (1987) suggest that the control of secretion of serum pIgA, like that of total serum IgA is quite independent of the secretion of intestinal IgA. In fact in the studies presented here the levels of pIgA antibody paralleled the levels of total serum IgA antibody. However, the serum pIgA response was transient. Whereas monomeric IgA antibody to typhoid LPS could be detected weeks after

either oral or parenteral typhoid vaccination the pIgA antibody response was short lived. Conley and Delacroix (1987) suggest that the appearance of pIgA antibody in serum serves as a marker of recent activation of IgA cells by antigen and suggest that the predominance of pIgA in babies' sera supports this hypothesis reflecting recent exposure to antigens. The short lived early serum pIgA response to oral or parenteral typhoid vaccination (Chapter 6) tends to support this.

If the appearance of pIgA antibody in serum serves as an indicator of recent activation of the immune system by antigen it could potentially have been utilised as an indicator of successful vaccination. However, the lack of correlation between the generation of intestinal and serum pIgA antibody demonstrated in this thesis meant that serum pIgA antibody could not be used as a correlate of the intestinal immune response in healthy subjects.

Although the results of this study indicate that the measurement of serum pIgA antibody would not be a reliable correlate of the intestinal immune response other methods of indirectly assessing the immunological priming of the intestinal immune system have been evaluated since the studies described in this thesis were begun. The detection of antibody secreting cells in the peripheral blood has provided a means of studying the response to oral

immunisation. Czerkinsky et al (1983) described a solid phase enzyme linked immunospot (ELISPOT) assay for evaluating antibody production by circulating cells. They used this assay (Czerkinsky et al, 1987) to demonstrate the presence of peripheral blood mononuclear cells spontaneously producing anti Streptococcus mutans antibody in volunteers who had ingested capsules containing killed Streptococcus mutans. Kantele et al (1986) have detected circulating cells committed to the secretion of specific antibody to S.typhi after oral vaccination of human volunteers with Ty21a. Kantele et al (1986) could not demonstrate a correlation between antibody secretion by circulating cells and antibody in saliva or faeces as they failed to detect anti typhoid antibody in the saliva or faeces of these volunteers. An ELISA which measured specific antibody secretion by peripheral blood lymphocytes which could be used to measure the immune response to oral vaccination with Ty21a was described by Forrest (1988). A correlation between the peak peripheral blood lymphocyte IgA antibody response and the degree of rise in IgA antibody in intestinal fluid was demonstrated (Forrest, 1988). These findings are yet to be correlated with protection against disease after oral vaccination.

Having successfully developed assays for both antigen specific pIgA and total serum pIgA and having examined the relationship of serum pIgA to intestinal IgA in healthy

subjects, the assays were used to examine the relationship of pIgA to intestinal IgA and to characterise the serum IgA antibody in disease in which there were reports of elevated levels of serum pIgA or total IgA or reports of altered intestinal immunity. Several important observations were made.

pIgA antibody to E.coli LPS was detected in the serum of patients with alcoholic liver disease but not in normal subjects and previous reports that these patients had elevated total serum pIgA (Andre and Andre, 1976; Kutteh et al, 1982b; Delacroix et al, 1983; Kalsi et al, 1983) were confirmed (Chapter 7). However, even in these patients with elevated levels of total serum pIgA and elevated levels of E.coli LPS specific serum pIgA, there was no direct relationship between the levels of intestinal IgA antibody to E.coli LPS and E.coli-specific serum pIgA, suggesting that the elevated levels of serum pIgA to E.coli LPS either reflected reduced clearance of serum pIgA by the liver or reduced clearance of E.coli antigens which enter the systemic compartment.

A group of patients with alcoholic liver disease who were given the oral typhoid vaccine Ty21a exhibited an impaired immune response compared to normal subjects (Chapter 7). Although some of the patients did mount a serum pIgA antibody response to the vaccine there was no

correlation between levels of typhoid-LPS specific pIgA antibody and intestinal IgA antibody after oral vaccination. This group of patients appeared to have an impaired intestinal immune response compared to normal subjects.

In addition to demonstrating that oral vaccination could generate serum pIgA antibody, evidence of a serum pIgA antibody response following enteric infection was found in patients presenting with Campylobacter enteritis (Chapter 8). Eight of the 13 patients studied but none of the controls had serum pIgA antibody to Campylobacter antigens. Unfortunately, the relationship between the serum antibody and intestinal antibody could not be examined in this study. However there was evidence of a close relationship between the levels of serum pIgA antibody to the Campylobacter antigens and the total serum IgA antibody to the antigens.

The lack of a relationship between intestinal IgA and serum pIgA was also demonstrated in the patients with coeliac disease (Chapter 8). Although the sample of patients was small, only 3 of the 6 patients with coeliac disease who had anti-gliadin antibody in intestinal fluid while ingesting gluten, had serum pIgA antibody to gliadin and intestinal anti-gliadin antibody was still present after a 6 month period on a gluten free diet although no

serum pIgA to gliadin could be detected in these subjects. Despite the lack of correlation between serum anti-gliadin pIgA and intestinal anti-gliadin IgA, pIgA antibody to gliadin was detected in patients with coeliac disease who were ingesting gluten but disappeared with removal of gluten from the diet. Similar results were reported by Mascart Lemone et al (1988) in a group of children with coeliac disease. The presence of anti-gliadin pIgA related to the presence of the antigen (gluten) in the diet. Intestinal anti-gliadin antibody was not examined. The presence of pIgA antibody to gliadin in patients ingesting gluten could be secondary to damage to the intestinal mucosa leading to a failure to secrete all the locally produced pIgA into the intestine or to the damage to the mucosa enabling the antigen to enter the systemic compartment. In the study reported in chapter 8 evidence for increased levels of total serum pIgA was found in the patients with coeliac disease with a trend to a decline in the level with gluten exclusion. This may reflect increased spillover of pIgA from the gut or increased production in the vascular compartment.

In the patients with Crohn's disease there was no elevation in either total serum pIgA or pIgA antibody to E.coli LPS although mucosal damage occurs in this disease. This finding might reflect the reported shift towards the production of monomeric IgA locally in the intestine

(Brandtzaeg and Korsrud, 1984; MacDermott et al, 1986).

The sample of patients with IgA nephropathy who were studied was small. In this group of patients with inactive disease, with no elevation in serum pIgA an increased pIgA reponse to mucosal immunistion was not seen. A study of a large group of patients at various stages of the disease would provide more information. The link between antigen stimulation at the mucosal surface and the disease could be explored more thoroughly if intestinal and serum antibody responses to oral vaccination were examined.

Further insight into the relationship of serum pIgA antibody to intestinal IgA antibody responses may be obtained by performing studies in patients with enteric infections where an intestinal antibody response occurs. Studies in patients recovering from Shigella dysentery or Rota virus infection where intestinal antibody responses have been demonstrated (Reed and Williams, 1971; Davidson et al, 1983) could be performed. Studies in patients with infections at other mucosal surfaces such as the respiratory tract may also provide insight into the control of the production of pIgA and its relationship to antigenic stimulation at mucosal surfaces.

In summary, a useful means of measuring both antigen specific and total pIgA in human serum has been established. These assays were used to demonstrate the appearance of antigen specific pIgA antibody in human serum following recent antigenic stimulation, either through the parenteral or mucosal route. The measurement of antigen specific pIgA in human serum did not directly reflect the production of IgA antibody in intestinal fluid and therefore could not be used as a reliable correlate of local intestinal immune responses. Future areas of study might include the investigation of the factors regulating the production and clearance of pIgA in diseases of the gastrointestinal and respiratory system.

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