HUMAN GASTROINTESTINAL MUCOSAL SECRETORY IMMUNITY: INVESTIGATION AND REGULATION

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To my mother and father, for their love and tolerance!.

Statement.

I declare that all the work contained in this thesis has been performed by myself unless otherwise stated herein.

Acknowledgment

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ABSTRACT

Current ideas of human gut immunity are derived heavily from animal studies; the few human studies have mainly addressed cellular aspects, and those on immunoglobulins and antibodies have used serum (or rarely jejunal aspirate) to investigate immune events at the gut level, assuming that these fluids are representative of the gut. The aims of this thesis were to develop, evaluate, and apply protocols for the study of gut secretions in man. Saliva was examined as a secretion in its own right, to investigate the relationship between systemic (serum) and mucosal antibodies, and as a potential mirror of immune events occurring more distally in the gut. Methods for the collection and processing of jejunal fluid, and intestinal fluid obtained via whole gut irrigation were then developed.

Enzyme linked immunosorbent assay techniques were used to measure total immunoglobulin concentrations and antibody levels to three representative dietary protein antigens, in saliva, intestinal fluid, jejunal aspirate, and serum. Healthy subjects and groups of patients with a variety of gut diseases likely to have increased immunity were examined.

There was great physiological variation in immunoglobulin concentrations and antibody levels in saliva, which were universally decreased by eating. In patients on a gluten-free or elemental diet, salivary antibody levels were maintained despite a lack of antigen stimulation. Neither saliva nor serum reflected immunity in gut lavage fluid,

the only regularly observed relationship being a positive correlation between serum and saliva, especially after gut mucosal damage had occured. Differences between control subjects and patients with coeliac disease were insufficient to allow the use of saliva as a diagnostic or clinical tool. Smoking strikingly influenced immunoglobulin concentrations, with a dose-dependent and reversible decrease in salivary IgA, and an increase in IgM. These alterations were not due to changes in the numbers of immunoglobulin-producing cells in the parotid gland. Smoking may also decrease IgA in lavage fluid.

Immunoglobulins and antibodies were regularly detected in lavage fluid. There were increased immunoglobulin concentrations and antibody levels in lavage fluid of both treated and untreated patients with coeliac disease and dermatitis herpetiformis, most markedly of IgM. There was no evidence of secretory immunodeficiency in patients with inflammatory bowel disease.

In conclusion, novel avenues of investigating gut immunity have been explored. There were marked variations in secretory immunity in healthy subjects and in patients with gut diseases known to have an immunological component. Neither saliva nor serum reflected gut immunity; therefore the gut should be studied directly.

Papers arising from this thesis

Where appropriate, formal permission to include papers has been obtained from the publishers and co-authors of the following papers.

Barton JR, Ferguson A. (1988) Do salivary antibodies to dietary antigens reflect inappropriate stimulation of GALT or hyperpermeability? In: Inflammatory Bowel Disease: current status and future approach. MacDermott RP (Ed). Amsterdam: Elsevier Science Publishers BV, 213-217.

Barton JR, Riad M, Gaze MN, Maran AGD, Ferguson A. (1990) Mucosal immunodeficiency in smokers and in patients with epithelial head and neck tumours. Gut 31, 378-382.

Barton JR, O'Mahony S, Ferguson A. (1990) Regulation of antibodies to food proteins in the common mucosal immune system: lack of correlation between antibody titres in saliva and intestinal fluid. In: MacDonald TT, Challacombe SJ, Bland PW, Stokes CR, Heatley RV, Mowat AMcI, eds. Advances in mucosal immunology. Lancaster: Kluwer Academic Publishers, 495-496.

O'Mahony S, Barton JR, Crichton S, Ferguson A. (1990) An appraisal of gut lavage in the study of intestinal humoral immunity. Gut 31, 1341-1344.

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CHAPTER ONE: INTRODUCTION, AIMS, AND APPROACH

INTRODUCTION

The study of the gut mucosal immune system in humans is relevant to a number of common and severe gastrointestinal diseases both in the developed countries and in the developing world. Aspects of gut immunity have wide-ranging implications in the aetiology, pathology, and treatment of various infective, inflammatory, and, perhaps, neoplastic diseases of the gut . The study of human gut mucosal immune responses has been hindered by practical difficulties, and ethical considerations, with respect to access to gut cells and secretions. Thus, current ideas of human gut immunity are derived heavily from animal studies, and it is widely acknowledged that animal models are frequently not representative of man. Human studies have mainly looked at histological aspects, and those on immunoglobulins and antibodies have mostly used serum to investigate immune events at the gut level, assuming that serum is representative of gut secretions. Only a few studies have used mucosal secretions, sometimes from outwith the gut or from small sections of gut, and have again assumed that these secretions are representative of the gut without any supportive evidence.

As the early stage of a programme of work in Edinburgh on the mucosal immune system in man, the broad aim of this thesis was to develop, evaluate, and apply protocols for the study of gut secretions. To begin with, saliva was

examined firstly as a secretion in its own right, secondly to investigate the relationship between systemic (serum) and mucosal antibodies, and thirdly as a potential mirror of immune events occurring more distally in the gut. As the studies progressed, two new topics emerged as meriting further work with respect to gut secretory immunity, smoking and inflammatory bowel disease.

APPROACH

Firstly, technical questions with regards to the type of saliva to be studied, the methodology and timing of salivary collection, and to the treatment and preservation of saliva, were answered. Three common food proteins were chosen as representative dietary antigens, and standard enzyme linked immunosorbent assay (ELISA) techniques employed to measure total immunoglobulin concentrations and antibody levels to the food protein antigens in saliva, intestinal fluid and serum. Groups of healthy clinical and laboratory staff were examined to establish reference ranges. Later on, groups of patients with a variety of gut diseases likely to have increased immunity were studied, namely those with inflammatory bowel disease, coeliac disease, or dermatitis herpetiformis. It became apparent that there was great physiological variation in immunoglobulin concentrations and antibody levels in saliva. In particular the influence of smoking on immunoglobulin concentrations was striking, prompting a detailed study.

Methods for the collection and processing of intestinal fluid obtained via whole gut irrigation were then developed using both healthy subjects and patients with a variety of gastrointestinal diseases. Initially I intended to use the ELISPOT technique to count numbers of gut mucosal immunoglobulin- and specific antibody-producing cells, obtained by biopsy from the small bowel at endoscopy or via Crosby capsule, as a "gold standard". However excellent results for immunoglobulin concentrations and antibody levels derived from assay of intestinal fluid diverted efforts away from the cellular aspects. Serum, saliva, and intestinal fluid were then obtained from healthy subjects, and a cross-sectional study on patients with inflammatory bowel disease was conducted. Serial salivary samples on patients beginning an elemental diet devoid of food protein antigens were also obtained.

Confirmation of the influence of smoking on salivary immunoglobulin concentrations prompted investigation into the mechanism of these effects. Study of numbers of immunoglobulin-producing cells in parotid gland specimens from normal parotid tissue obtained at surgery was undertaken using immunoperoxidase staining techniques.

STRUCTURE

The thesis begins in section I with a general review of salivary and intestinal secretory immune physiology and pathophysiology; the literature on healthy smokers, as related to this thesis, is emphasised in the relevant chapter later on. The basic methodologies and the technical developments and experiments, in particular the protocols for collection, processing, and assay of saliva and intestinal fluid are described in this section.

In section II the physiology of salivary and intestinal fluid immunoglobulins and antibodies in healthy subjects and in patients with coeliac disease and dermatitis herpetiformis is discussed, as is the relationship between serum, salivary, and intestinal immunoglobulins and antibodies. In section III the effects of smoking on salivary immune parameters in two very different populations from Edinburgh and Cairo are described. The alterations in immunoglobulins and antibodies in the cross-sectional study of patients with inflammatory bowel disease are reported. Finally the thesis is concluded with a general discussion of the findings, and future studies prompted by the work.

CHAPTER TWO: REVIEW OF SALIVARY AND GUT IMMUNE PHYSIOLOGY

Introduction

The gut immune system is comprised of all mucosal and exocrine tissues from the mouth to the rectum, of which the salivary glands are thought to be a part. There have been many recent and excellent reviews of the components, anatomy, and physiology of the gut associated lymphoid tissues and these will not be repeated here (Brandtzaeg et al, 1985; Doe, 1989; Elson, 1985; Elson et al, 1986; Marsh, 1987). In this chapter I will confine my review to aspects of the anatomy, development, and physiology of salivary and gut secretory immunity in man directly relevant to the subject matter of my thesis.

SALIVARY GLANDS

Immunohistology

Secreting either directly or via a duct into the mouth are three major pairs of, and many minor, salivary glands. The parotid, sublingual and submandibular glands comprise the three major pairs of glands. All have similar ratios of immunoglobulin isotype-producing cells, although the density of those cells may vary between glands (Korsrud and Brandtzaeg, 1980). In man IgA-producing cells predominate, with 80-90% of all immunoglobulin-producing cells dedicated to this isotype. The remainder of the cells are IgM, IgG, and IgD producers with between one and six percent each, IgE-producing cells are virtu-

ally absent (Korsrud and Brandtzaeg, 1980; Beckenkamp, 1985). The many scattered minor salivary glands have similar isotype ratios to the major glands and may have greater density of IgA- containing cells (Matthews et al, 1985), although IgA cell numbers may be equalled by those of IgM (Deslauriers et al, 1985) and these glands may have a substantial role in oral immunity. A comprehensive review by Nair and Schroeder clearly presents firm evidence for this role, and supports Mesteckys' theory that minor salivary glands may react directly to topical antigens (Mestecky et al, 1978), which have gained local access through the salivary ducts as well as participating in the immune response of the common mucosal immune system (Nair and Schroeder, 1986). Animal studies have demonstrated that the complete range of cells necessary for an immune response is present within salivary glands.

In IgA deficiency, IgM producing cells may predominate (Brandtzaeg, 1971a), although in one patients' parotid glands IgD-positive plasma cells increased, with the proportions of cells being 20%, 27%, and 53% for IgM, IgG, and IgD respectively (Korsrud and Brandtzaeg, 1980).

At least one group consider that immunocyte numbers in parotid salivary glands may not reflect the secretory immunoglobulin content of parotid or of mixed saliva, as the parotid glands have a comparatively low density of immunocytes, and immunoglobulin isotype percentages may not be a reflection of the global oro-pharyngeal immuno-

cyte population (Beckenkamp, 1985). Others have shown good correlation for immunoglobulin isotype concentration between parotid saliva and labial saliva when sampling healthy subjects. Their study may have been flawed by the use of semi-quantitive, comparative, methodology, and their use of autopsy specimens raises the possibility of autolytic artefacts. They also suggested that the isotype distribution of the B cells in the secretory tissue may not be a reflection of the salivary immunoglobulins secreted (Deslauriers et al, 1985).

Immunoglobulin valency and production

Most IgA-positive plasma cells in salivary glands produce dimers or larger polymers, as evidenced by an 84% cytoplasmic affinity rate for secretory component, and a 92% J-chain positivity. Both of these percentages are reduced in inflammation; Brandtzaeg and Korsrud considered that the former reduction suggested a switch to proportionally greater monomeric IgA production and that the latter suggested clonal maturation (Brandtzaeg and Korsrud, 1984). They advance little evidence to support this hypothesis, comparisons with dissimilar tissues forming the basis of their arguments. Secretory IgA in saliva is largely dimeric, usually 90% or more (Delacroix et al, 1982; Smith et al, 1987b), although Delacroix does not state if whole or parotid saliva is studied, and the number of subjects studied is small (n=7). After production within the immunocyte, transcellular uptake, transport, and secretion through and from the acinar cells is

likely to be a physiological parallel of that seen across the gut epithelium (Nakamura et al, 1985; Ahnen et al, 1985).

IgA subclasses

All secretions contain proportionally more IgA of subclass 2 when compared with serum, (e.g. salivary mean percentage IgA2 37±13%, intestinal perfusate 30±5%, serum [range + maximum standard deviation] 18-21±8%) (Delacroix et al, 1982); this may alter during the course of a secretory immune response to certain bacterial antigens (Streptococcus pyogenes, Bacteroides fragilis and E. coli lipopolysaccharide [LPS]) when there may be a further increase in the proportion of IgA2, in contrast to other bacterial infection (Strep mutans glucuronyltransferase [GTF]) or candidal infection where IgA1 may similarly increase, both proportionally and in absolute amount (Brown and Mestecky, 1985; Jeganathan et al, 1987). In this latter study the IgA2 subclass dominance of anti-Candida albicans antibodies in health was reversed by oral infection (Jeganathan et al, 1987). In another study, specific anti- pneumococcal antibodies of both IgA subclasses increased equally after pneumococcal vaccination (IgA1 x2.4, IgA2 x2.0), with no increase in total IgA. A much greater increase was seen for IgG (x4.5) and IgM (x4.0) specific antibodies in saliva (Lue et al, 1988). Thus the nature of the antigen may determine which of the two subclasses the corresponding IgA antibody will be composed of, and it seems that in parot-

id saliva a specific antibody response may be largely associated with either IgA1 or IgA2 (Brown and Mestecky, 1985).

DEVELOPMENT OF SALIVARY IMMUNITY

Immunoglobulins are detectable in saliva shortly after birth, with IgA being found in 50% of full-term neonates, but only 8% of pre-term infants by 8-14 days. These rates of detection increase to 83% and 63% respectively by 15-21 days, with serum IgA becoming detectable a few days later (Haworth and Dilling, 1966). The investigators used cotton- wool to collect parotid saliva however, and acknowledged potential contamination by mixed saliva and loss of IgA by absorption onto cotton-wool by this method. They also used the insensitive radial immunodiffusion technique for immunoglobulin determination. A more recent study using ELISA detected specific IgA and IgM antibodies to E. coli O antigen and to poliovirus type I on day one of life in neonates. These antibodies were found in saliva of a child whose mother had hypogammaglobulinaemia (Mellander et al, 1986). A comprehensive longitudinal study, using mixed saliva, of 165 infants followed for 5 years demonstrated low (<1 ELISA unit) levels of antibodies to E. coli for the first 4 years of life (Gleeson et al, 1987a). Low levels of salivary IgM were detected at four weeks, and IgD was present in an inverse concentration to IgA. It was thus rarely detected after six months of age (Gleeson et al, 1987b). A further well-conducted study by this group suggested a maturation

sequence for IgA in saliva. Sucrose density gradient centrifugation was used to study the molecular form of IgA; they demonstrated that whilst in the first year of life both monomeric and dimeric IgA could be detected in saliva, that by the second year only the dimeric form was found. Secretory component was always present, but was not biochemically linked to the monomeric IgA present (Cripps et al, 1989).

Further maturation of salivary immunity occurs through childhood, with children at 12 to 17 months having higher whole salivary IgA concentrations than those aged 12 months or less. This may be linked to the eruption of teeth; the use of whole saliva unfortunately prevents clear interpretation due to the possibility of crevicular leakage (Gahnberg et al, 1985). A study of 1,539 children suggested that IgA was generally lower than in adults, but reached adult levels at age 6 years; unfortunately IgG and IgM were not assayed (D'Amelio et al, 1986). Most studies concur that adult salivary immunoglobulin concentrations are reached by four to six years of age, and specific antibody titres somewhat later at five to nine years (D'Amelio et al, 1986; Tenovuo et al, 1987; Gleeson et al, 1987a).

Influence of breast feeding

Breast feeding increased total salivary IgA and IgM levels, and specific IgA antibody levels to E. coli, at 6 days in 15 breast-fed infants, but not salivary IgG or

IgM antibodies to E. coli, nor any isotype of antibody to tetanus toxoid when compared with 15 bottle-fed infants. By 6 weeks however the antibody levels in the two groups of infants were similar (Stephens, 1986). Others have failed to find any influence of breast feeding on the ontogeny of the salivary immune response (Gleeson et al, 1982), or found that bottle-fed infants had higher salivary IgA levels (Ostergaard, 1985). Unfortunately these studies were also carried out on whole saliva.

Ageing and salivary immunity

There is a large literature on the influence of ageing on systemic immune parameters but few studies of its effects on secretory immunity. The published work is almost exclusively concerned with salivary immunity. In a small study, reported in abstract only, using insensitive radial immunodiffusion techniques on unstimulated saliva obtained after breakfast, total salivary IgA and IgG levels were not different between 10 young and 10 old subjects; IgM was not detected (Ganguly et al, 1987). Others have demonstrated a fall in total IgA concentration in human nasal secretions with age (Alford, 1968). Whilst IgA subclasses in parotid saliva did not change with ageing in a comprehensive study by Smith et al, they did find low levels of specific antibody to S. mutans GTF and poliovirus in elderly humans (70-91 years) when compared with young adults. This may reflect changes in the number or function of T and/or B cells, or simply decreased antigen exposure (Smith et al, 1987a). Dimin-

ished salivary and nasal secretory responses to influenza immunisation have been noted in a well-designed study comparing young adults with adults aged >60 years. Whilst this may be interpreted as decreased capacity for immune response, (Waldman and Bergmann, 1987) it may reflect the higher pre- existing specific antibody levels found in the saliva and nasal secretions of elderly subjects which might exclude the pathogens. Furthermore, the "elderly" subjects were not particularly elderly.

In summary, there is general agreement that secretory immunoglobulins and antibodies in saliva become detectable in the neonatal period, and slowly mature to adult levels by age 4-9 years. There is no conclusive evidence of any change in salivary immunity in a healthy aged population.

PHYSIOLOGY OF SALIVARY IMMUNOGLOBULINS

Relationship to salivary flow and proteins

There are many studies demonstrating the marked variation in salivary protein concentrations and composition, and these have been briefly reviewed (Dawes, 1981). Although IgA secretion rate and concentration has been meticulously studied by Brandtzaeg, and reported in a classic series of papers, (Brandtzaeg et al, 1970; Brandtzaeg, 1971a and 1971b) neither total IgG nor IgM, nor antibodies of any specificity have been subject to such scrutiny. Brandtzaeg was also limited by the relatively crude methodology available at that time, and found it necessary to concentrate saliva forty times using acrylamide rods after addition of merthiolate preservative, and sometimes to further concentrate, by lyophilization, prior to assay by single radial immunodiffusion. Use of sensitive, modern ELISA techniques removes the need for any of these processing steps with their concomitant estimated loss of IgA of at least 20% (Brandtzaeg et al, 1970), indeed for this thesis samples of saliva needed dilution rather than concentration to achieve optimum assay conditions (chapter 4).

Despite these caveats it seems clear from these and other studies that salivary IgA concentrations are inversely correlated with salivary flow rate (Mandel and Khurana, 1969; Brandtzaeg, 1971b). This inverse relationship seems to hold true when prolonged stimulation or collection of

saliva is undertaken, but no data exists on sampling limited to a few minutes. Brandtzaeg took continuous whole and parotid salivary samples for up to 105 minutes from seven subjects and showed that when flow rate increased salivary IgA concentration decreased, but that the rate of IgA secretion increased. Salivary flow was simulated by lemon juice, and serial samples over an hour using this technique gave consistent results for IgA concentration (Brandtzaeg, 1971b).

The relationship of IgA with total salivary protein or components such as lactoferrin, peroxidase, and lysozyme is weak or absent (Brandtzaeg, 1971b; Rudney and Smith, 1985), and the authors conclude that IgA should not be expressed as a function of total protein present.

Effects of food and eating

Only a single small study using insensitive methodology has addressed the effect of eating itself on salivary IgA concentration; IgA concentration fell after a meal (South et al, 1966). A further small study which is nevertheless unique studied the influence of regular ingestion of a food (yogurt) on the levels of antibodies to two components of that food (Streptococcus thermophilis and Lactobacillus bulgaricus). Antibodies were present in both groups but no differences in IgA, IgG, or IgM antibody levels in saliva (or urine) were found when comparing yogurt eaters with non-eaters, although serum IgG antibodies to S. thermophilis were higher in the yogurt

eaters. Antibody levels to Streptococcus lactis were studied before and after eating a fermented milk product containing the bacterium. Antibodies were present prior to ingestion, and did not change in titre (Carlsson and Bratthall, 1985). One interpretation of these data is that the bacterium was excluded from the gut by these antibodies and was thus unable to stimulate the mucosal immune apparatus; this explanation is also suggested by several studies discussed later (Clancy et al, 1983; Waldman et al, 1986; Waldman et al, 1987).

Differences between whole and parotid saliva

When studying saliva the choice of which type of saliva to collect is limited to either whole (mixed) saliva, or to parotid saliva for practical reasons . Several authors have demonstrated that whole saliva is partly comprised of small but significant amounts of serum-derived crevicular fluid (which includes, of course, serum immunoglobulins) which seeps out at the gingival margins (Brandtzaeg et al, 1970; Izutsu at al, 1985; Lindstrom and Folke, 1973). This contamination, especially when there is inflammation in the oral cavity, may lead to high IgA and IgG concentrations in whole saliva. A comparison of whole salivary immunoglobulin concentrations in healthy elderly people with and without teeth found that those subjects without teeth had 80% less total IgG than their dentate peers. Unfortunately parotid saliva was not analysed (Gronblad and Lindholm, 1987). Parotid saliva is not susceptible to contamination by

crevicular fluid and, although the authors debate about the real contribution of crevicular leakage to whole salivary immunoglobulin and antibody concentrations, the consensus view is that parotid saliva is the purer secretory fluid.

Physiological variation in salivary immunoglobulins and antibodies

There are conflicting studies on the day-to-day variation in secretory immunoglobulin concentrations and specific salivary antibody levels between individuals. Unstimulated and, even moreso, stimulated salivary IgA levels in children showed good correlation over both a short (10 minutes) and long (two years) period in one study (Brathall and Widerstrom, 1985). Unfortunately whole saliva was collected, and, where appropriate, secretion was stimulated by the subjects chewing paraffin gum, a substance which absorbs immunoglobulins (Brandtzaeg, 1973). In another study IgA in whole saliva and parotid saliva was subject to marked variability in sequential samples taken from individuals at weekly intervals (Rudney et al, 1985). In both studies there was good correlation between immunoglobulin concentrations in stimulated and unstimulated saliva. The only paper which assessed specific antibody variation over a prolonged period (4 months) demonstrated marked fluctuations in antibody levels to oral streptococci in 12 serial samples of parotid saliva. Antibody levels correlated with each other, and with total IgA concentration in the five subjects studied in

this small but important paper. Interestingly parotid saliva had greater antibody activity than whole saliva (Gahnberg and Krasse, 1981).

Circadian and hormonal influences

A large prospective analysis of whole salivary IgA concentrations in 289 children over three years showed no seasonal fluctuations, although IgA did vary considerably within an individual. It would have been valuable to sample saliva more than twice a year in order to exclude significant alterations (Wagner et al 1982). A much smaller study of the influence of the time of year on salivary IgA in nine subjects did find fluctuations but was flawed methodologically in several areas. Paraffin gum chewing was used to stimulate whole salivary flow. This may increase crevicular leak and may absorb immunoglobulins from the saliva (see above). Different durations of salivary collection were employed, and parotid salivary samples were collected for unspecified periods until a certain volume of saliva had been obtained, immediately after collection of whole salivary samples. The study showed that both for whole and parotid saliva, IgA concentration is lower in springtime than in autumn, and non-specifically increased in pregnancy, especially in the third trimester and early post-partum period (Widerstrom and Bratthall, 1984). A further study on hormonal variations throughout the menstrual cycle did not show any effect on salivary immunoglobulin concentrations (Bratthall and Widerstrom, 1984). Others have shown

a decrease in salivary IgA in the premenstrual phase of the cycle in man (Kubitz et al, 1986). No other gender-related differences have been reported, and a study of 1,539 subjects demonstrated no difference in IgA concentrations between male and female children (Aguirre et al, 1987).

Stress

Stress has repeatedly been shown to be associated with a decreased salivary IgA, as is "negative" mood, whilst a "positive" mood, relaxation, and happiness are associated with an increase in salivary IgA concentrations (Jemmott et al, 1983; Dillon et al, 1985; Green and Green, 1987; Kubitz et al, 1986; Stone et al, 1987). In a number of the studies correlation of decrease in IgA with stress (within subjects) was poor, and definitions of stress were loose. No account was taken of tobacco or alcohol use (Jemmott et al, 1983; Stone et al, 1987). However the uniformity of results in the papers, studying either whole or parotid saliva and using different types of stress, supports the relationship. The decrease in salivary IgA concentration may occur shortly after the end of the stressful period, may be preceded by a transient increase, and may be related to high catecholamine levels generated by the stress (McClelland et al, 1985).

Exercise

Severe exercise transiently reduces salivary IgA and IgM, the effects lasting less than 24 hours. Along with other

described immune alterations this supports anecdotal statements by athletes of an increased susceptibility to illness during intense training (Tomasi et al, 1982; Mackinnon et al, 1987).

Nutrition

In studies on the effect of nutritional status on salivary immunity, diminished IgA concentrations were only found in those children who were severely malnourished and in whom gut and other infections and infestations were present (Watson et al, 1985). Another study of malnourished adults found an increased concentration of salivary IgA and IgG. Cotton-wool was used for salivary collection, which took much longer in the malnourished group than the controls. Most of the patients had neoplastic disease or chronic inflammatory disease whereas the controls largely had ischaemic heart disease. Like the first study, radial immunodiffusion was used to quantify immunoglobulins (Cheatham et al, 1984). Thus the effect of pure malnutrition on salivary immunity has not been studied.

SALIVARY IMMUNITY AND ORAL INFECTION

Introduction

The interpretation of studies on the effects of secretory immunity on oral infection must be tempered with the knowledge that just as in the more distal gastrointestinal tract, defence mechanisms are multiple and include other proteins such as lactoferrin, peroxidase, lysozyme, an intact mucosa, and salivary flow. Support for this multifactorial scheme comes from several studies, confirmed and reviewed by Tenuvuo (Tenuvuo et al, 1987).

Dental caries

Conflicting reports of salivary total immunoglobulin concentrations and of specific antibody levels in dental caries exist. Salivary antibodies may protect the oral cavity; IgA anti-S. mutans antibodies in parotid saliva decreased numbers of S. mutans bacteria found in both dental plaque and in whole saliva following 10 days of oral immunisation with killed S. mutans bacilli (Gregory and Filler, 1987). A detailed survey of antibodies to seven S. mutans serotypes in 12 volunteers suggested that dental colonisation by S. mutans species was inversely proportional to specific salivary IgA antibody levels (Gregory et al, 1985). Other work described above supports this finding (Smith and Taubman, 1987a). A high natural level of parotid salivary IgA anti-S. mutans ribosomal antibody correlated in healthy individuals with decreased susceptibility to dental caries (Gregory et al, 1986).

However others have found that caries-susceptible children had a more rapid increase in whole salivary total IgA concentrations in the second year of life compared with controls, although levels fell towards those of the caries- free controls by 4 years of age (Alaluusua, 1983). In a study which measured parotid salivary total IgA by single radial immunodiffusion, high IgA concentrations were inversely correlated with the incidence of caries, and positively related to gingival inflammation (Orstavik and Brandtzaeg, 1975). Unfortunately the use of total immunoglobulin levels rather than antibodies, and of whole saliva, render interpretation of many of these reports difficult (Bruno et al, 1985).

Upper aero-alimentary infection

In oral candidiasis specific salivary anti-Candida albicans IgA antibody was increased, falling with treatment of the infection towards normal. No details of the methodology or timing of collection, nor the type of saliva are given (Jeganathan et al, 1987). Total salivary IgA was decreased in the acute phase of infectious mononucleosis (Marklund et al, 1984), although to confirm the theory that mucosal immunoincompetence leads to susceptibility to infectious mononucleosis, sampling should really have been prospective and have included data from subjects not acutely ill.

A remarkably low mixed salivary total IgA concentration was found in children prone to upper respiratory tract infections (Lehtonen et al, 1987). Salivary IgA levels

when the children were healthy were inversely correlated with the number of upper respiratory tract infections contracted in one year in another study of children, with salivary IgA levels rising considerably during an acute respiratory infection (Isaacs et al, 1984). A decreased salivary IgA level in patients with active graft-versus host disease may explain why these subjects are predisposed to sino-bronchial infection (Izutsu et al, 1983; Izutsu et al, 1985).

Experimental induction of salivary antibodies

There have been a number of recent studies on the effects of natural exposure to or vaccination with various bacterial and viral antigens on salivary antibody response. An oral killed polyvalent bacterial vaccine containing Haemophilus influenzae and Staphylococcus aureus, given on three occasions over two months resulted in an increase in antibodies to H. influenzae in the parotid saliva of 55% of subjects compared with 6.7% of controls taking placebo. The antibodies were mainly IgA, and there was no change in total salivary immunoglobulin concentrations, in specific salivary antibodies to a control antigen (E. coli), or in serum antibodies. The presence of high pre-immunisation antibody levels was inversely correlated with the antibody response to vaccination (Clancy et al, 1983).

Natural exposure of infants to poliovirus resulted in salivary antibodies detectable at one month of age, reaching adult levels by six months. Vaccination with

either live attenuated or inactivated virus by mouth required three doses before antibodies were detected, with a further increase in levels after a fourth vaccination. Not surprisingly, natural exposure seemed more efficient at inducing a response than vaccination (Carlsson et al, 1985). In a study flawed by the use of whole saliva stimulated by chewing paraffin gum (see above), the above results were reinforced in an investigation of natural cholera infection and oral vaccination with B subunit-whole cell vaccine. Following natural infection >80% of patients had a rise in salivary specific antibodies compared with 44% after two vaccinations (Jertborn et al, 1986), [see below for more details].

Other studies have demonstrated the independence (from systemic immunity) and kinetics of the salivary immune response. A well-conducted study of 14 subjects and 11 controls showed a rise in parotid salivary specific antibodies after an intensive schedule (13 doses in the first regime, followed by 5 doses in the second regime) of oral vaccination with a Streptococcal GTF combined with aluminium phosphate as an adjuvant. No changes occurred in serum antibodies. No rationale is given for the large number of immunisations however, and only one control group, immunised with aluminium phosphate, was used. A second group of controls given streptococcal GTF would have allowed assessment of the usefulness or otherwise of the adjuvant effect of the aluminium phosphate (Smith and Taubman, 1987a).

In a well-designed but small study (n=8) both IgA1 and

IgA2 antibodies were induced in parotid saliva and other secretions by oral vaccination with killed S. mutans given daily for ten days. The antibodies persisted for >50 days. Seven further days vaccination resulted in a more rapid and greater response which persisted longer than previously (Gregory and Filler, 1987). Furthermore in these last two studies there was an effect on both S. mutans reaccumulation (Smith and Taubman, 1987a) and viability in dental plaque (Gregory and Filler, 1987).

Three papers report on the comparative efficacy of salivary antibody induction via parenteral and oral routes. A killed pertussis vaccine was given either orally or parenterally on days 2, 3, 4, and 5 after birth, followed by a booster dose at 42 days in over 20,000 neonates. Oral vaccination led to IgA specific antibodies in whole saliva in the majority of children, whereas parenteral vaccination did not result in any salivary antibodies (Baumann et al, 1985). Salivary antibodies in whole saliva in a group of children who received oral poliovirus vaccination at two months were compared with a group who had two parenteral vaccinations at 9 and 10 months. At age one year, 26% of the orally vaccinated group had antibodies, compared with 9% of the parenterally vaccinated group. Unfortunately the groups were from different countries (North America and Scandinavia), and the parenteral group was small, 13 subjects, (Smith et al, 1986). A double-blind, placebo-controlled, trial of oral enteric-coated versus intra-muscular killed influenza A

vaccine demonstrated a rise in salivary (and nasal) IgA specific antibodies after four weeks only in the orally vaccinated group. Once more, pre-immunisation antibody levels were inversely correlated with the post-vaccination antibody rise (Waldman et al, 1986).

Two reports suggest memory for both cellular and humoral components of the local IgA immune response at the mucosal level to both bacterial and viral antigens (Ebersole et al, 1983; Wright et al, 1983).

Correlations between antibodies in saliva, other secretions, and serum

A paper describing antibody responses to acute rotavirus infection in the serum and duodenal secretions of ten children reports that one week after their diarrhoeal illness, there was a significant rise in IgM specific antibodies in both serum and secretions. In the convalescent phase four weeks later IgA and IgG antibodies were present in serum, and only IgA antibodies in duodenal secretions. Ten children with bacterial enteritis acted as control subjects (Davidson et al, 1983).

Gut lavage was used to obtain samples of intestinal fluid for assay of four antigens of enterotoxigenic Escherichia coli in 15 patients with acute watery diarrhoea due to the bacterium. Serum, saliva, and breast milk were also assayed. The majority of patients had a greater than two-fold increase in their intestinal fluid antibody levels, and associated rises in saliva and breast milk antibody levels. However, the relationships between these incre-

ments were not clear (Stoll et al, 1986).

A key paper, briefly alluded to above, assessed antibody responses in whole saliva, breast milk, and serum, and made comparisons with intestinal secretions obtained by saline lavage of the gut following oral cholera vaccination or natural disease (Jertborn et al, 1986). There were a few relatively minor methodological problems; heat-treated whole saliva was used, and intestinal lavage was heat-inactivated and concentrated, which may account for the poor reproducibility in duplicate testing of samples. The numbers of patients who responded with a rise in antibody levels restricts interpretation of the data, some of which (correlations) would have been more appropriately analysed by non-parametric methods. Despite these criticisms the findings remain important. After three immunisations, 13 of 15 subjects had a rise (> 2.3-fold) in antibodies to cholera toxin in gut lavage fluid, and 7 of 15 subjects had a rise (>1.5-fold) in salivary antibodies. The rise in salivary antibodies had a sensitivity of 53% and 50% in predicting a rise in intestinal antibody levels after one and two immunisations respectively, with positive predictive values of 77% and 88%. IgA specific antibodies to cholera in saliva correlated with IgA antibodies in intestinal lavage after both the first ($r=0.48$, $n=25$) and second vaccinations ($r=0.51$, $n=18$; $p<0.05$ for both). Furthermore, lavage IgA antibodies correlated with both serum IgA ($r=0.4$, $n=25$, $p<0.05$) and serum IgG antibodies ($r=0.55$, $n=21$, $p<0.01$) after two immunisations.

In a study reported in brief, antibodies to S. mutans (a common oral commensal bacterium) were measured in tears and parotid saliva and found to be of similar levels, the conclusions being that a common mucosal immune system regulated levels from a distance (Allansmith et al, 1983). A further paper from the same group reverses the original conclusions. Antibody levels were higher to S. mutans in parotid saliva than in tears, whilst antibody levels to Staphylococcus epidermidis (a skin commensal) were the reverse of this pattern. The final conclusion was that local antigen concentrations regulate the final level of antibodies in secretions (Gregory and Allansmith, 1986). However this thesis founders on the assumption that the parotid gland is local; unless antigen travels up the parotid duct from the oral cavity, induction of antibodies by oral antigens must be indirect. The high serum titres found also suggest that systemic immunisation has occurred and this may bias analysis from the mucosal standpoint.

Thus salivary antibody function may parallel that in more distal intestinal secretions, reducing viral, bacterial, and fungal colonisation and infection as referred to above (Baumann et al, 1985; Czerkinsky et al, 1987; Holt et al, 1987; Kurz et al, 1986; Jertborn et al, 1986; Smith et al, 1986; Waldman et al, 1986).

SALIVARY IMMUNOGLOBULIN DEFICIENCY

IgA is the serum immunoglobulin most commonly deficient, with approximately one in 700 adults with low or undetectable levels (Haeney, 1990). The equivalent data for salivary IgA is derived from three studies. In two studies from the same group, published two years apart, the incidence of salivary IgA deficiency was 3 of 1000 adult males by radial immunodiffusion using a 7S standard (D'Amelio et al, 1982), and none of 315 adult males using a more sensitive nephelometric assay (D'Amelio et al, 1984). No details of the method of salivary collection are given. In infants the incidence of salivary IgA deficiency fell from 6% at 4 months of age to 1% at 20 months, where the lower limit of detection was 5mg/L (van Asperen et al, 1985).

Salivary IgA deficiency may predispose to an increase in the frequency and severity of atopy (Taylor et al, 1973). Salivary IgA deficiency was more frequent amongst the infants of atopic parents when compared with children of non-atopic parents, but within the former group absence of whole salivary IgA did not result in a greater incidence of clinical atopy (Van Asperen et al, 1985). Atopy in the infants, who were aged four to 20 months, was based on reported symptoms and skin prick testing, both of which may not be more than 50% specific or sensitive (Lehmann, 1980). However, others have also found that salivary IgA concentration is low in atopic children with a diagnosis of asthma, independent of steroid treatment (Maeda,

1985).

In assessing any possible effects of salivary IgA deficiency it seems important to measure specific antibody levels as well as total immunoglobulin concentrations. In salivary IgA deficiency peak serum immune complex levels after a milk challenge were inversely related to the titre of antibodies to milk proteins in saliva, but not to total serum or salivary immunoglobulins (Cunningham-Rundles et al, 1979).

SMOKING AND IMMUNITY

The effects of smoking on the systemic immune system have been extensively studied, and recently were comprehensively reviewed (Holt, 1987). In particular, it seems that smoking is associated with a decrease in serum levels of immunoglobulins A, G, and M, and, generally, increased IgE and IgD concentrations (Burrows et al, 1981; Burrows et al, 1982; Gerrard et al, 1980; McSharry et al, 1985; Robertson et al, 1984; Warren et al, 1982), some reports dissenting (Friedhoff et al, 1986). Where studied, these alterations were found to be reversible. Natural killer cell activity is reduced (Ginns et al, 1985; Sopori et al, 1985), certainly in heavy smokers (Phillips et al, 1985). Total T cells and both T_h and (to a lesser extent) T_s cells are increased in moderate smokers, with heavy smokers in contrast showing a decrease in the $T_{h/s}$ ratio, although T_s function was impaired when assayed with PWM stimulated cells (Burton et al, 1983; Hughes et al, 1985). Again these effects were thought to be reversible. In uterine cervical epithelium there were reduced numbers of macrophages in smokers (Barton et al, 1988).

Surprisingly studies directed at exploring the effects of smoking on the mucosal immune system are scarce. There have been a few studies on total immunoglobulin concentrations in saliva. A small (n=8), highly selected, group of elderly edentulous males had decreased whole salivary IgA, comparisons between other groups of smokers and non-

smokers failing to demonstrate any difference in IgA concentrations. No standards were used in the assays, of which some were radial immunodiffusion and some immuno-turbidimetric because of instrument unavailability (Bennett and Reade, 1982). A further study limited by size and inappropriate statistics showed a trend towards an increase in whole salivary IgA on stopping smoking which failed to reach significance, (Hersey et al, 1983). Others used paraffin-chewing to stimulate whole salivary flow, but failed to find any differences in IgA concentrations between smokers and non-smokers, nor any effect of nicotine chewing gum alone on salivary IgA in a large investigation (Olson et al, 1985). A paper on secretory IgA concentrations in oropharyngeal carcinoma also assessed smoking and non-smoking controls using two methods of collection for whole saliva, 1) simple expectoration for 15 minutes, followed by 2) saline oral washing. In the expectorated samples from smokers low IgA levels were observed, whereas in the washings high concentrations were present when compared with the non-smoking controls. The groups were small (9 smokers, 8 controls), four of the smokers had severe periodontal disease, and the controls were subjects with miscellaneous oto-rhino-laryngological conditions including allergic and infective diseases. The patients with carcinoma had high concentrations of IgA in their saliva, which was mainly secretory (Watanabe et al, 1983).

Few papers have addressed the effects of smoking on either serum or secretory antibody responses to mucosal-

ly- encountered antigens. The studies which have done so have generally recorded a diminished serum antibody response to antigens encountered at mucosal surfaces. Antigens associated with pigeon breeders lung were assessed in the serum of pigeon breeders in central Scotland. Serum antibodies were detectable in one of 23 smokers, compared to 39 of 65 non-smokers, with an increase in antibody levels in ex-smokers in this well-conducted study (McSharry et al, 1985). Similarly 14 of 15 non-smokers but only 13 of 41 smokers, all working in the same room in a cigar factory, had serum antibodies to bacterial antigens found in the humidifier water from that room and, importantly, this was independent of acute respiratory symptoms. Nevertheless it is still impossible to exclude bias due to pre-existing respiratory problems (Andersen et al, 1982). Titres of serum antibodies to enterically-encountered antigens (C. albicans and E. coli) were not different between the groups. Minor criticisms were that total and not isotype-specific antibodies were estimated, and the statistical analysis was qualitative, not quantitative. It is suggested that the lack of an effect of smoking on these latter two antibodies is because the two antigens do not enter the body through the respiratory mucosa where smoking is likely to have its most profound effect.

The only report on mucosal antibodies in smokers suggests a decreased production of antibodies to cotton dust bacterial antigens (Pseudomonas and Enterobacter) in

nasal secretions of wood and cotton workers at two mills. Although IgG and IgA antibodies were lower in smokers at both mills, this difference was only significant for IgG antibody levels in the workers from the wood mill. Once more numbers were small, and importantly it is not stated how many cigarettes were smoked (Rylander et al, 1982).

Two papers assessed the effects of smoking on oro- pharyngeal colonisation with commensal organisms. Increased concentrations of Lactobacilli and S. mutans were found in the whole saliva of smokers, numbers of lactobacilli correlating with cigarettes smoked per day (Heintze, 1984). A large study found that although smokers had the same carrier rate for Candida albicans in the mouth and saliva, that those who were colonised had double the number of colony-forming units compared with the non-smoking carriers. However the overlap of results between the smoking and the non-smoking groups was wide, and the differences failed to reach statistical significance, (Oliver and Shillitoe, 1984).

Of relevance are studies of immunoglobulins in respiratory tract secretions. Although studies of saliva have found a decrease in secretory immunoglobulins, IgA and IgG were elevated three- or four-fold in the broncho-alveolar lavage fluid of 6 smokers (manual/farming workers, mean age 39.5 years, all males) when compared to 6 non-smoking students (mean age 23 years, three males). Other proteins in the lavage fluid were unchanged, sug-

gesting a true local increase in production. No IgM was detected. This may be a reflection of the insensitivity of radial immunodiffusion assay (Velluti et al, 1983). In contrast, mucosal immunodeficiency induced by smoking has been demonstrated by decreased IgA levels in broncho-alveolar lavage (Gotoh et al, 1983). Others have failed to demonstrate any difference in IgA concentrations, but found an increase in IgG (Warr et al, 1977).

A study of cells in broncho-alveolar lavage fluid obtained from volunteer non-smokers and smokers show that the latter have diminished numbers of OKT4+ and increased numbers of OKT8+ cells, the changes correlating with total smoke dose. In peripheral blood there were higher leucocyte, total lymphocyte, and OKT8+ cells in the smokers, but no significant change in the OKT4/OKT8 ratio. In contrast in lavage the alteration in the ratio of OKT4/OKT8 cells was highly significant (Costabel et al, 1986).

Two recent studies provide further evidence of the effects of smoking on local aero-alimentary immunity. In a case-controlled study of naso-pharyngeal meningococcal carriage during an outbreak of meningococcal disease, smoking significantly increased the carriage rate. The more cigarettes smoked, the greater the odds ratio, especially above 20 cigarettes daily. Unfortunately secretory antibodies to meningococcus were not measured (Stuart et al, 1989); diminished local antibody production may have

accounted for the increased carriage rate. A study of smoking habits in platinum refinery workers demonstrated convincingly that smoking increases, in a dose-dependent manner, the risk of developing occupational sensitisation to platinum salts. Platinum is known to induce occupational asthma, although the study addressed skin tests and respiratory symptoms, and not reversible airways obstruction. However smokers, especially of more than 10 cigarettes daily, were much more likely to develop a positive skin test, respiratory symptoms, and to leave employment because of these symptoms than were non-smokers (Venables et al, 1989).

Summary

There is a reversible decrease in serum IgA, IgG and IgM, and an increase in IgD and IgE in smokers. There are diminished serum (and possibly secretory) antibody responses to antigens encountered via mucosal surfaces. There is an increase in the carriage rate or colonisation of aero-alimentary pathogens in smokers. There are no real data on the influence of smoking on salivary immunoglobulin concentrations, and conflicting data on immunoglobulin concentrations in respiratory tract secretions.

INTESTINAL FLUID PHYSIOLOGY

Introduction

There has been little or no systematic study of immunoglobulins or antibodies in intestinal fluid in vivo in man. The few reports which exist have mainly used jejunal

aspirate as the gut secretion. One group has used saline to lavage the gut, and another assayed solid faeces. Most studies are descriptive, and provide no information on basic gut secretory physiology. All reports can be faulted in one or more of several areas: small numbers of subjects, heterogeneous groups of both controls and patients, failure to use protease inhibitors (immediately), the use of insensitive assays, and inappropriate statistical analysis. Nevertheless, they are the only reports available, and as such are summarised below.

Cells and secretions

Early studies on duodenal aspirate universally demonstrated the prescence of IgA but failed to detect IgM or IgG in 80% of the samples. This may have been because of insensitive methodology, or a failure to use protease inhibitors, (eg. Plaut and Keonil, 1969). There is common consensus that intestinal fluid IgA and IgM are locally produced (Jonard et al, 1984), but debate as to whether or not IgG and IgD are locally or plasma derived (Hjelt et al, 1988). Virtually all studies have found that IgA is present in the highest concentration, followed by IgM and IgG. More recently others have detected IgD in small amounts (Hjelt et al, 1988), and IgE-producing plasma cells have been demonstrated in gut mucosa, although molecular IgE itself has not been found in secretions (Savilahti, 1972).

Intestinal IgA was found to be present both as a dimer

and monomer, just as is the case with saliva (Bull et al, 1971).Intestinal IgA increases in concentration with age, at least in childhood, with children under two years having lower concentrations than older children (Savilahti, 1972). In a study, on faeces, of a miscellany of diseases presenting with diarrhoea, the ratio of monomeric to total IgA increased, as did total IgA concentrations (Meillet et al, 1987). Adults have higher ratios of polymeric to monomeric IgA which correlates positively with polymeric IgM concentrations (Hjelt et al, 1988). There seems to be little variation with time in IgA concentration in freeze- dried stools in a sequential study, a single sample correlated well (r=0.84) with the mean of a daily complete stool collection and concentrations seemed to be stable over several days (Haneberg and Aarskog, 1975). No data are available on either intestinal immunoglobulins or antibodies in elderly humans.

Effects of eating, and of infection

The effects of a meal on duodenal and upper small bowel immunoglobulin concentrations has only been subjected to a cursory study in four volunteers, levels increasing in two and decreasing in the other two subjects (Samson et al, 1973). On the effects of intestinal infection however there is consensus. IgA concentrations increase in intestinal fluids or faecal samples after gastroenteritis due to salmonella (LaBrooy et al, 1980; LaBrooy et al, 1982; Hoj et al, 1984), Yersinia (Fletcher et al, 1988), rotavirus (Davidson et al, 1983), shigella (LaBrooy et al,

1980; Keren et al, 1988), experimentally-induced and naturally acquired cholera infection (Jertborn et al, 1986), and in fact any infectious gastroenteritis (Haneberg and Aarskog, 1975) or diarrhoeal disease (Meillet et al, 1987).

With gut infection, IgM antibody levels rose quickly but were not sustained, although as might be expected, the specific IgA antibody concentrations were maintained (Davidson et al, 1983). Antibody titres showed enormous variation both in initial magnitude of response and in rate of decline, with antibody being detectable for as little as two weeks or as long as one year after acute infection (LaBrooy et al, 1980). One group perfused isolated jejunal loops in vivo to assess the antibody response to typhoid vaccination. A specific antibody response was induced in all 14 subjects, and was present three weeks later (Bartholomeusz et al, 1990). There was no correlation between serum and intestinal antibody levels.

Correlations between antibodies in lavage and in other fluids.

Studies addressing the correlation between immunoglobulin concentrations and antibody titres in intestinal fluid with values in other secretions have given variable results. No correlation with parotid saliva was found in one study, although groups were heterogeneous and small in number, and may have been at different stages of gut infection (LaBrooy et al, 1980). Whole gut saline lavage

was used to obtain intestinal fluid in order to assess gut secretory antibody response after natural cholera infection or vaccination. IgA specific antibodies were induced in gut lavage fluid in the majority of subjects, and parallelled by a rise in IgA antibody titres in whole saliva ($r=0.51$, $p<0.05$), in breast milk, in serum ($r=0.4$, $p<0.05$), and by IgG antibody titres in serum ($r=0.55$, $p<0.01$), (Jertborn et al, 1986).

Generally, although the relative numbers of IgA, IgM, and IgG gut plasma cells are reflected by the concentrations of immunoglobulin isotypes in gut fluid, no-one has satisfactorily demonstrated any correlation between the two parameters (see below).

Investigation of intestinal immunity

Only recently has a pilot study using a safe, non-invasive technique been described which allows assessment of intestinal secretory immunity. A non-absorbable polyethyleneglycol-electrolyte lavage solution, taken orally allows measurement of immunoglobulins in gut fluid, and is safe in patients with renal, hepatic, and cardiac disease (Gaspari et al, 1988).

Inflammatory bowel disease

Little information exists on secretory immunoglobulin concentrations in patients with inflammatory bowel disease. Levels of IgA were normal, but IgG was high in the freeze- dried faeces of patients with quiescent ulcera-

tive colitis (Haneberg and Aarskog, 1975). There were no differences detected between controls and 36 heterogeneous patients with Crohn's disease when jejunal aspirate was assayed, and no correlation between jejunal aspirate immunoglobulin concentrations and in vitro jejunal biopsy immunoglobulin production (Jones et al, 1976). In one other study resected small and large bowel specimens from nine patients with Crohn's disease were perfused with saline at 4^{oC} for 24 hours. There was a higher recovery of IgG from involved mucosa when compared with uninvolved bowel (Bergman et al, 1973).

In contrast many reports exist on the mucosal cell populations and in vitro immunoglobulin production in inflammatory bowel disease. Despite some controversy, generally they demonstrate increased numbers of all isotypes of plasma cells, with a proportionally greater increase in the number of IgG cells in involved mucosa (Brandtzaeg and Baklien, 1976; Rosekrans et al, 1980; Scott et al, 1984). Findings in ulcerative colitis seems to be similar to those in Crohn's disease (Bookman and Bull, 1979; Soltoft, 1969)

Coeliac disease

In patients with coeliac disease jejunal aspirate IgA, IgG and IgM are reported to be increased in one report (Lancaster-Smith et al, 1974), with another study only able to demonstrate an increase in IgM concentrations (Douglas et al, 1970).

Insensitive tests which failed to discriminate between isotypes showed that antibodies to food proteins are detectable in the intestinal fluid of patients coeliac disease (Ferguson and Carswell, 1972; Katz et al, 1968; Mawhinney and Love, 1975). Recently, using sensitive ELISA techniques, two further reports have given conflicting results. One suggests that antibody levels in intestinal fluid may be correlated with the degree of intestinal damage (Volta et al, 1988), whereas the other study does not find this (LaBrooy et al, 1986). Antibodies to foods can also be found in normal children however (Ferguson and Carswell, 1972). In patients with coeliac disease, jejunal aspirate antibodies to a gluten fraction and to ßlactoglobulin had not significantly fallen six months after changing to a gluten-free diet, whilst serum antibody levels did drop (LaBrooy et al, 1986).

Again, more information exists on mucosal plasma cell populations and immunoglobulin production, the consensus of which suggests an increase in IgA and IgM plasma cells (Ciclatira et al, 1986; Douglas et al, 1970; Scott et al, 1984; Wood et al, 1986; Wood et al, 1987), with the occa sional report extending that finding to IgG cells (Wood et al, 1987).

Summary

IgA is the predominant immunoglobulin in gut secretions,

followed by IgM and IgG. All isotypes increase in a number of gut diseases, such as infection, inflammatory bowel disease, and coeliac disease, perhaps with IgG increasing proportionally more in active IBD and IgM in coeliac disease. A specific antibody response to oral infection/vaccination can be detected in gut fluids. Few correlations have been satisfactorily demonstrated between gut fluid immunoglobulins or antibodies when assaying serum or other mucosal secretions, with one or two exceptions. Little is known about the basic physiology of intestinal secretory immunity in vivo, especially in healthy subjects.

CHAPTER THREE: SUBJECTS AND STANDARD METHODOLOGY

INTRODUCTION

In this chapter the origin and documentation of the subjects who participated in the studies is described. Standard methodologies in widespread use, which needed little or no modification for the purposes of the thesis, are also described.

SUBJECTS

Pilot studies and technical experiments.

Serum and salivary specimens for the pilot studies were obtained, with informed consent, from healthy laboratory and clinical medical staff without noting details of age, sex, or social habits, and similarly from in-patients on the Gastrointestinal unit. Permission from the Lothian area ethical committee had been obtained for the collection of serum and saliva.

Salivary and intestinal secretory physiology.

1. Healthy volunteers.

To describe the basic physiology of parotid saliva, healthy volunteers were recruited from laboratory and clinical medical staff. All volunteers were taking a normal diet containing the food protein antigens under study, and were not abusing alcohol or taking any medications likely to influence the studies. Demographic details were recorded on a standard proforma (appendix ix), which included an alcohol and smoking history, a

dietary history with specific questions on intake of bread (to allow an approximate guide to the intake of gliadin), milk (for ßlactoglobulin), and eggs (for ovalbumin), a history of any medical problems and a list of any drugs taken. Demographic data on these healthy volunteers are given in table 3.1.

Table 3.1 **Details of healthy subjects - saliva studies**

Subjects	**Sex**		**total**	**age**		**smokers**
	M (n)	**F (n)**	**(n)**	**median**	**(range)**	
Study						
18 hr variation	6	3	9	27	(21-31)	1
alcohol	5	3	8	25.5	(23-32)	2
dietary antigens						
eggs - Y	8	13	21	40*	(18-92)	4
- N	7	6	13	23	(14-84)	1
bread- Y	8	10	18	34.5	(18-81)	4
- N	7	8	15	25	(14-84)	0
milk - Y	8	11	19	36	(18-92)	4
- N	6	5	11	23	(14-84)	0

Age, sex, and smoking habits of control subjects, from whom samples were obtained in the studies of salivary physiology concerning 1) 18 hour variation during a single day, 2) after a single dose of alcohol, and 3) in subjects including (Y) or not including (N) eggs, bread, and/or milk in their diets. Key: * = p=0.04, (Mann-Whitney test) subjects taking egg significantly older than those without egg in diet. There were no other significant differences in age, sex, or smoking habits between the groups (Chi-squared and Kruskal-Wallis tests).

2. Healthy patients.

Some of the studies on the physiology of immunoglobulins and antibodies in saliva, serum, and intestinal fluid were conducted using patients investigated by the gastrointestinal unit for clinical problems. Patients were deemed healthy and included in the study if at the end of clinical and laboratory assessment they had a diagnosis of functional disease, or of minor clinical medical problems, unlikely to influence the immune parameters being studied. Patients with infective, immune, inflammatory, or neoplastic disease were excluded. Full demographic details were recorded as above, including the patients' final diagnosis. Salivary and serum samples were obtained on all subjects, and in addition if clini-

Table 3.2 **Details of healthy subjects - lavage studies**

Subject	Sex M (n)	Sex F (n)	total (n)	age (yrs) median	age (yrs) (range)
Controls					
Volunteers					
non-smokers	4	6	10	29	(22-56)
smokers	5	7	12	32	(23-56)
Miscellaneous					
all	9	16	25	53	(21-92)
Coeliacs					
untreated	6	9	15	43	(28-62)
treated	8	11	19	46	(16-72)

Demographic details of subjects participating in study of physiology of lavage fluid. The groups of control subjects were derived from healthy volunteers, and from healthy patients (see text for details). There were no significant differences for age and sex distribution between the groups, (Kruskal-Wallis test).

cal investigation included a barium enema or colonoscopy patients were asked if they would have lavage as a rapid bowel preparation (as detailed in chapter 4) rather than the standard two day laxative regime of sodium picosulphate, codanthromer, and oral fluids only. If clinical investigation included a jejunal biopsy patients were asked to consent that aspiration of jejunal contents could also be carried out. Details of these healthy patients are included in tables 3.1 and 3.2.

3. Patients with dermatitis herpetiformis.

In a collaborative study conducted by Dr. Carolyn McDonald from this laboratory, in conjunction with Dr David

Table 3.3 **Subject details - salivary physiology**

Subject	Sex M (n)	F (n)	total (n)	age median	(range)	smokers
controls	13	15	28	25.5	(14-34)	6
coeliac disease						
- untreated	14	18	32	41.5	(16-78)	9
- treated	14	20	34	42.0	(15-72)	7
dermatitis herpetiformis						
- ND, NH	8	4	12	58.0	(27-86)	2
- ND, P/SVA	8	3	11	64.0	(15-83)	2
- GFD, NH	11	8	19	49.0	(28-82)	7
- GFD, P/SVA	15	3	18	52.5	(26-77)	5

Age, sex, and smoking habits of control subjects, and patients with coeliac disease or dermatitis herpetiformis, from whom samples were obtained in the study of salivary physiology. Key: ND = normal diet, GFD = gluten-free diet, NH = normal jejunal histology, P/SVA = partial or sub-total villous atrophy. No differences between groups (Kruskal-Wallis test).

Gawkrodger from the department of Dermatology at the Royal Infirmary of Edinburgh, a well-defined cohort of patients with dermatitis herpetiformis was being investigated.

Table 3.4 **Subject details - correlation studies**

Subject		Sex M (n)	F (n)	total (n)	age (yrs) median	(range)
Controls						
ser/sal vs	- lav	9	16	25	53	(21-92)
	- asp	13	15	28	25.5*	(14-84)
Coeliac disease						
Untreated						
ser vs	- sal	14	18	32	41.5	(16-78)
ser/sal vs	- asp	12	14	26	39.5	(16-78)
	- lav	6	9	15	43	(28-62)
Treated						
ser vs	- sal	14	20	34	42	(15-72)
ser/sal vs	- asp	9	13	22	42	(15-72)
	- lav	8	11	19	46	(16-72)
Dermatitis herpetiformis						
NH, GFD		10	11	21	52	(27-82)
P/SVA, ND		12	7	19	54	(15-83)
P/SVA, GFD		17	4	21	52	(26-80)
Heterogeneous subjects						
		10	14	24	45	(22-72)

Legend: Demographic details of subjects used in study of relationships within the systemic and mucosal immune systems. Key - ND = normal diet, GFD = gluten-free diet, NH = normal jejunal histology, S/PVA = subtotal or partial villus atrophy; ser = serum, sal = saliva, asp = aspirate, lav = lavage. Controls having jejunal aspirate younger than other groups, * $p<0.05$, Mann-Whitney test.

On request, samples of saliva were also collected from these patients in addition to the serum already obtained for the original study. All patients had had a dietary history for gluten taken by a trained dietitian and a recent jejunal biopsy, in addition to other details on the standard questionnaire as for the healthy patients above. Clinical details are given in tables 3.3 and 3.4.

4. Patients with Coeliac disease.

Patients presenting with a new diagnosis of, and those with established and treated, coeliac disease who were undergoing jejunal biopsy for clinical purposes, were asked to participate in the study and to give salivary and serum samples at the time of jejunal biopsy. Clinical details were recorded on the standard proforma. These patients were also invited to undergo gastrointestinal lavage for the purposes of the study on intestinal secretions. Clinical details are given in tables 3.3 and 3.4.

5. Smoking and epithelial head and neck tumours.

In the first phase of this topic of study, patients with the following disorders attending the relevant outpatient clinics at the Royal Infirmary of Edinburgh were recruited as non-gastrointestinal disease controls: treated hyper- and hypothyroidism, obesity, bulimia nervosa, and patients with epithelial head and neck tumours both untreated and after a course of radiotherapy. These patients were recruited with the help of Dr Magdy Riad, an otolaryngology research fellow, in a collaborative

study. A control group of healthy clinical, laboratory, and technical staff including both smokers and non-smokers were also studied. These control subjects were not age- and sex-matched for the patient groups, and only brief demographic details were recorded.

In the second phase of this study, patients newly presenting with epithelial tumours of the oropharynx, hypopharynx, and larynx to the outpatient clinics of clinical and radiation oncology at the Western General Hospital were studied prospectively prior to and following a course of radiotherapy. Dr Mark Gaze, registrar in radiation oncology, identified patients and obtained the samples. Brief details including smoking habits, dentition, clinical status, and treatment regimen were recorded.

In the third phase of this study, to confirm the findings from the previous phase, it was necessary to recruit subjects who smoked but abstained from alcohol. In collaboration with Dr. Riad, who collected the samples, healthy non-smokers, smokers, and ex-smokers from a non-drinking population were recruited from clinical, laboratory and ancillary staff, and from patients attending for the removal of ear wax at the Ain Shames University Hospitals, Cairo, Egypt. In addition, all patients with epithelial head and neck tumours who were attending the Department of Otolaryngology in these hospitals were invited to participate. Healthy subjects and patients did

not take alcohol, and non-smoking groups were separately age- and sex-matched for healthy smokers and for the

Table 3.5 **Subject numbers and sex - smoking study**

	Edinburgh			**Cairo**		
Subjects	M	F	total	M	F	total
Healthy controls						
non-smokers	31	83	114	19	11	30
smokers	19	9	28	27	20	47
ex-smokers -2yr	-	-	-	11	10	21
ex-smokers -5yr	-	-	-	10	7	17
age + sex matched for tumour patients	-	-	-	18	10	28
Tumour patients						
untreated						
-current smokers	13	4	17	14	4	18
- ex-smokers	?	?	?	7	4	11
- non-smokers	?	?	?	0	1	1
- unknown	5	5	10	0	0	0
total	18	9	27	21	9	30
irradiated						
-current smokers	0	0	0	7	2	9
- ex-smokers	?	?	?	14	7	21
- non-smokers	?	?	?	0	0	0
- unknown	26	12	38	0	0	0
total	26	12	38	21	9	30
Other malignancies						
smokers	-	-	-	4	1	5
non-smokers	-	-	-	7	7	14
total	-	-	-	11	8	19

Sex distribution of subjects from whom parotid saliva was obtained for the study of the effects of smoking on salivary immunoglobulins. Key: ? = not recorded, - = not recruited. No differences were found when comparisons were made between appropriate groups (Kruskal-Wallis test).

Table 3.6 **Subject age - smoking study**

Healthy controls	**Edinburgh**	**Cairo**
non-smokers		29.2 (21-36)
smokers		29.8 (21-37)
ex-smokers -2yrs	-------	33.8 (27-44)
ex-smokers -5yrs	-------	55.1 (36-78)
age + sex-matched for tumour pts	-------	51.6 (34-61)
Tumour patients		
prospective group	65.7 (49-83)	-------
upper aero-alimentary tract		
untreated	56.1 (41-69)	52.7 (31-64)
irradiated	56.4 (31-81)	59.0 (48-73)
other malignancies (Cairo)	-------	53.1 (43-81)

Median age (range), in years, of subjects from whom parotid saliva was obtained for the study of the effects of smoking on salivary immunoglobulins. No significant differences were found when comparisons were made between appropriate groups (Kruskal-Wallis test).

tumour patients. Finally, a group of age- and sex-matched patients with tumours outwith the head and neck region were recruited from the same hospital. Details of the site of the tumour in both these and the head and neck tumour patients were recorded, and are shown in table 3.7. A control group of patients with tumours outwith the head and neck were also recruited for comparison. Clinical details of all of the subjects are recorded in tables 3.5, 3.6, and 3.7.

Patients who had received radiotherapy had been treated with a standard 52.5 Gy course in 20 treatments over a mean of 30.14 (±5.8) days, range 26-50. The field of

irradiation did not include the parotid glands.

Table 3.7 **Tumour origin in patients - smoking study**

Site of tumour	**untreated**	**irradiated**
Prospective - Edinburgh		
larynx	13	--
pharynx	1	--
Prospective - Cairo		
upper aero-alimentary tract		
larynx	13	11
hypopharynx	9	8
oropharynx	8	11
total	30	30
other sites		
bladder	7	-
colon	3	-
cervix	4	-
uterus	2	-
prostate	2	-
bone	1	-
total	19	

Sites of primary tumours in patients from whom parotid saliva was obtained in the study of the effects of smoking on salivary immunoglobulin concentrations

The final phase of this aspect of mucosal immunity involved obtaining parotid gland specimens. They were taken from cadavers undergoing routine post-mortem examination at the Western General Hospital with the collaboration of Dr Margaret McIntyre (consultant pathologist), and from operative specimens of parotid glands of patients undergoing parotidectomy for parotid adenoma in the City

Hospital, the Royal Infirmary, and the Western General Hospital, Edinburgh. The specimens were kindly collected by Professor AGD Maran from the department of Otolaryngology.

6. Inflammatory bowel disease.

Patients were recruited from the new and review gastrointestinal outpatient clinics at the Western General Hospital, from those attending the gastrointestinal investigation suite, and from inpatients on the gastrointestinal and surgical wards. For all patients full demographic details were recorded on the standard proforma as above. Serum and parotid saliva were obtained from all patients and, as for coeliac disease, if clinical assessment was to include barium enema or colonoscopy, or the patients were due to have surgery necessitating bowel preparation, they were invited to have that preparation via gut lavage as above (and see chapter 4). Clinical details are given in table 3.8.

Clinical questionnaire.

A questionnaire was completed for all subjects, (except where stated for the pilot studies), healthy volunteers as well as patients. The format of this questionnaire was altered at one stage, after the influence of smoking, alcohol and diet had been appreciated. The questionnaire is shown in appendix (ix) and records age, sex, diet and drugs as well as details of health status, smoking, alcohol intake, and dentition.

Table 3.8 **Subject details - inflammatory bowel disease study**

Subject		Sex M (n)	F (n)	total (n)	age (yrs) median	(range)
Controls		9	13	22	30.5	(22-56)
Crohn's disease						
active	- NS	5	2	7	28	(17-62)
	- S	3	8	11	40	(10-66)
total		8	11	18	34.5	(10-66)
inactive	- NS	1	1	2	41.5	(37-46)
	- S	2	3	5	36	(16-65)
total		3	4	7	37	(16-65)
Ulcerative colitis						
inactive	- NS	12	6	18	51	(27-80)
	- S	6	5	11	44	(22-55)
total		18	11	29	46	(22-80)

Demographic details of volunteer subjects, and patients with inactive and active Crohn's disease, and patients with inactive ulcerative colitis, subdivided by smoking habits. Key: NS = non-smokers, S = smokers. There were no significant differences in age and sex between the groups (Kruskal-Wallis test).

Universal comments and exclusions.

Full informed consent was obtained in every case. Prior to collection of saliva in every subject, the oral cavity was examined clinically for evidence of local sepsis (overt periodontal sepsis, pharyngitis, tonsillitis, dental abscess), if any of these conditions were present, the person was excluded from the study and saliva was not collected. Similarly, unless specifically stated, any

subject with immunological, neoplastic, inflammatory, or an active infective disease was excluded from study.

METHODS

Introduction.

Standard methods for the measurement of total protein and total antibody activity in body fluids are well established. For the purpose of this thesis, simple, safe, reliable and reproducible techniques were required, preferably ones which needed little in the way of extra equipment. The Pierce modification of the Lowry method for protein assay was already being used in this laboratory for other purposes.

1. Protein assay.

The Pierce method uses Bicinchoninic acid (BCA) as a protein assay reagent (Pierce (UK) Ltd, 36, Clifton Road, Cambridge). It is based on the reaction of protein with alkaline copper $^{2+}$ which produces copper^{1+}. The stable, soluble reagent reacts with this copper^{1+} to form an intense purple-coloured solution at 562nm, two molecules of BCA complexing with one cuprous ion. The standard protocol is fully described in appendix (i).

The Pierce protein assay is a recent modification of the Lowry method, offers several advantages over the original, and was thus chosen to assay total protein concentrations in saliva and intestinal fluid. By eliminating

vortexing and precise, timed reagent addition the Pierce modification is simpler than Lowry's method, is more sensitive, has increased compatibility with non-ionic detergents, and has a wider linear working range.
The Pierce method gave reproducible results and excellent correlations for the standard curves. In the pilot study, salivary protein concentrations were a mean 1.684 ± 0.468 (sd), range 0.98-2.54mg/ml (n=26). Although lavage fluid gave similarly reliable results for protein concentrations, the addition of various protease inhibitors and fetal calf serum to the specimens required to inhibit immunoglobulin and antibody degradation (see chapter 4) added exogenous protein to the assay and prevented interpretation of total protein concentrations.

2. Enzyme linked immunosorbent assay of serum antibodies.
An enzyme linked immunosorbent assay (ELISA) technique had also been previously used to measure total serum antibody activity to three common food protein antigens in humans in this laboratory, and was currently in use for the measurement of isotype-specific serum antibodies to ovalbumin in a mouse model. This working assay was adapted for use in these studies. Test ranges of assay reagent dilutions were carried out and minor modifications were made. The final protocol is described in appendix (ii).

Absorption and dilution studies confirmed the specificity of the antibody detection (appendix ii). Variation of the

assay results is given in chapter 4. Although there are several methods suitable for the measurement of total immunoglobulins and antibodies, the enzyme-linked immunosorbent assay (ELISA) was chosen as the standard technique for the analyses performed for this thesis. All equipment necessary was available, as was previous experience with the assay. The other advantages of ELISA were its high sensitivity, adaptability, and reproducibility. The assay was also fairly simple to perform, and avoided the use of radioactive reagents with their hazards and need for caution which the chief rival methodology, radioimmunoassay, did not.

3. Measurement of total serum immunoglobulins.

The availability of a routine assay in the hospital clinical biochemistry department for total immunoglobulin A, IgG and IgM levels in serum meant that these assays could be conveniently carried out by that department with their kind agreement, and the permission of Dr G Blundell. The immunoglobulin levels are assayed by immunoturbidimetry, using a Monarch centrifugal fast analyser, with two dilutions to check for antigen excess, and using a British working standard for human serum immunoglobulins G, A, and M (67/99) as a reference standard (National Institute of Biological Standards, Potters Bar, Herts). Results were expressed in iu/ml.

4. Collection of saliva and sterilisation of apparatus.

The collection and processing of parotid saliva and

lavage was largely carried out using disposable equipment where possible, to reduce time spent on cleaning and to guarantee sterlility. In some instances however it was necessary to re-use equipment. When re-usable equipment was used to obtain patient and volunteer samples disinfection became important. The only equipment which came into this category for the purposes of this thesis were the Carlsson- Crittenden cups. Other non-disposable items used in the collection and assay procedures were cleaned and disinfected in the standard manner, but were not in contact with subjects and the cleaning procedure will not be described.

Sterilisation of Carlsson-Crittenden cup.

After collection of saliva, the cup itself was cleaned under the tap in running water to remove any gross contamination by secretions. The tubing was then flushed through with tap water at high velocity using a full 60 ml. syringe for each tube. The tubes were cleared of water with 60 mls. of air passed through at high pressure again with a hand-held syringe. The whole apparatus was then immersed in 100% ethanol, ethanol was aspirated through the tubing with a syringe, and the entire assembly left to soak for 15 minutes submersed in the ethanol. The apparatus was then removed from the ethanol, air dried using a 60 ml syringe as previously described, and left to dry out fully between adsorbent paper overnight. Paired swabs were taken from the cup, and saline lavage of the tubing obtained from 20 Carlsson-Crittenden cups

after sterilisation. These samples were sent for bacteriological and virological culture. No significant growth of bacteria, nor detection of viruses was found. The method for cleaning and disinfecting the Carlsson-Crittenden cups proved entirely satisfactory and the concentration of alcohol and the immersion times were both well in excess of the minimum recommended by local infection control standards.

5. Immunohistology.

To identify isotype-specific plasma cells in parotid glands, a standard immunoperoxidase technique was applied to both post-mortem and per-operative samples. This is detailed in appendix (iii).

Reasonable preservation of architecture, with lymphocytes with positive staining for IgA, IgG and IgM were found. There was significant background staining on post-mortem specimens, making identification of plasma cells more difficult, but fresh, per-operative specimens were free from this problem. The standard protocol for immunoperoxidase staining for other tissues worked well without any modifications being made, and resulted in slides adequate for plasma cell counting. The numbers of plasma cells of each isotype were enumerated by direct visual counting of cells under a light microscope per ten high-power fields for each specimen.

6. Cellobiose-mannitol permeability test.

To assess intestinal permeability during an alcohol challenge, the above test was used. It is based upon the principle that sugars of differing molecular sizes are absorbed at differential rates, and are dependent on different aspects of the gut mucosa. The absorption of a small molecular sugar is proportional to the surface area of the epithelium of the gut, whilst the absorption of a large molecular sugar is inversely proportional to the integrity of the mucosa. Both sugars used are excreted unchanged in the urine.

After an overnight fast, the two sugars (cellobiose - large molecule, and mannitol - small molecule) are dissolved in 200mls of water and drunk after the bladder has been voided. Nothing is taken by mouth for two hours, after which subjects can have water ad libitum. All urine is collected, and a final voiding of the bladder is requested at the end of the test at five hours after ingestion of the sugar solution. The urine sample volume is measured, and the sugar concentrations determined by enzymatic methods, with the use of a spectrophotometer. A ratio of the two sugars in the urine is derived and compared to a reference ratio for our laboratory. A value above this range suggests an increase in gut permeability.

7. DATA STORAGE AND PROCESSING

The computer hardware and software used in the preparation of this thesis are given in appendix (iv), along with comments as to their suitability and limitations.

8. STATISTICS

Statistical analyses were carried out using a number of different methods. The non-parametric Mann-Whitney U test was used for all unpaired comparisons of groups of results. Student's t-test and Wilcoxon's paired rank sum test were used for paired data, and either the Kendall (on the Amstrad computer, using the Amstat statistical software programme) or the Spearman (using the Minitab statistical software programme on the IBM-compatible computers) rank correlation coefficients for non-parametric regression analysis. The Chi-squared test, with Yates correction when small numbers were being analysed, was used for qualitative data. Where multiple comparisons between groups were being made, oneway analysis of variance (for parametric data) or the Kruskal-Wallis test (for non-parametric data) were performed initially. If a significant difference was found, then comparisons between individual groups were made by the Mann-Whitney U test. A probability value of $p=0.05$ or less was taken as being significant, however where many statistical tests were performed with regards to one set of analyses, only a p value of less than 0.01 was regarded as significant (this applies particularly to chapter 8).

CHAPTER FOUR: TECHNICAL DEVELOPMENTS AND EXPERIMENTS

Introduction

Having decided to collect pure parotid saliva on the grounds that it was a pure secretory fluid, it was necessary to establish the best method of salivary collection, and to determine whether or not the methodologies developed for ELISA analysis of immunoglobulins and antibodies in serum were applicable to saliva. The specificity and reproducibility of these ELISA assays were also determined. Parotid saliva was treated with a mucolytic, with a detergent, and by sonication in attempts to increase the recovery of immunoglobulins at assay; the possibility of contamination by crevicular fluid leaking in between the collecting cup and the buccal mucosa was also assessed. The other major aims at this stage were to establish a method for the collection of whole gut intestinal fluid by gut lavage, and to establish methodologies for the assay of total immunoglobulins and antibodies in gut lavage fluid.

SALIVA

Approach

In practical terms there were two possible methods for collecting parotid saliva, cannulation of the parotid duct being discounted on the grounds of invasiveness and time required, making the method unsuitable for use as an outpatient procedure. The two remaining methods were 1) to use an occlusive cup placed over the parotid duct or

2) to use absorbent material such as cotton-wool or a dental roll, placed between the upper second molar tooth (opposite the parotid duct), and the cheek. The use of such absorbent material would be simpler, faster, and possibly as good as using a cup. Therefore bilateral samples of parotid saliva using both methods were simultaneous collected over five minutes in 26 healthy volunteers.

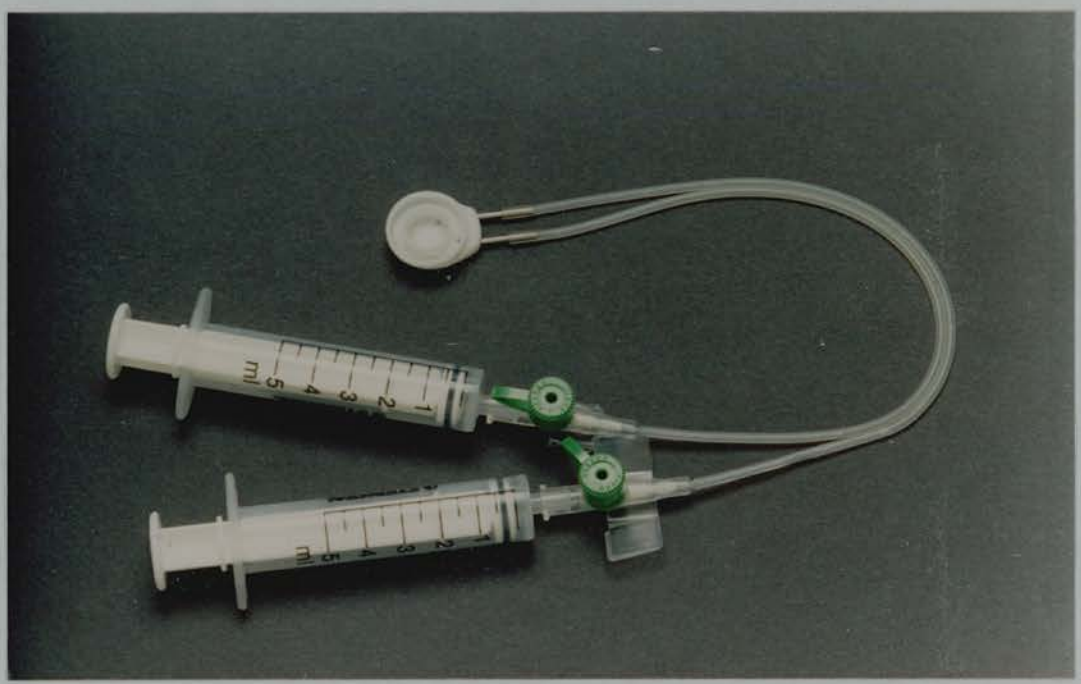


Figure 4.1 - Carlsson-Crittenden cup

Parotid salivary flow was stimulated by 5% citric acid, given by disposable pipette to the floor of the mouth in 0.5 ml aliquots each minute, to a total of two mls. A

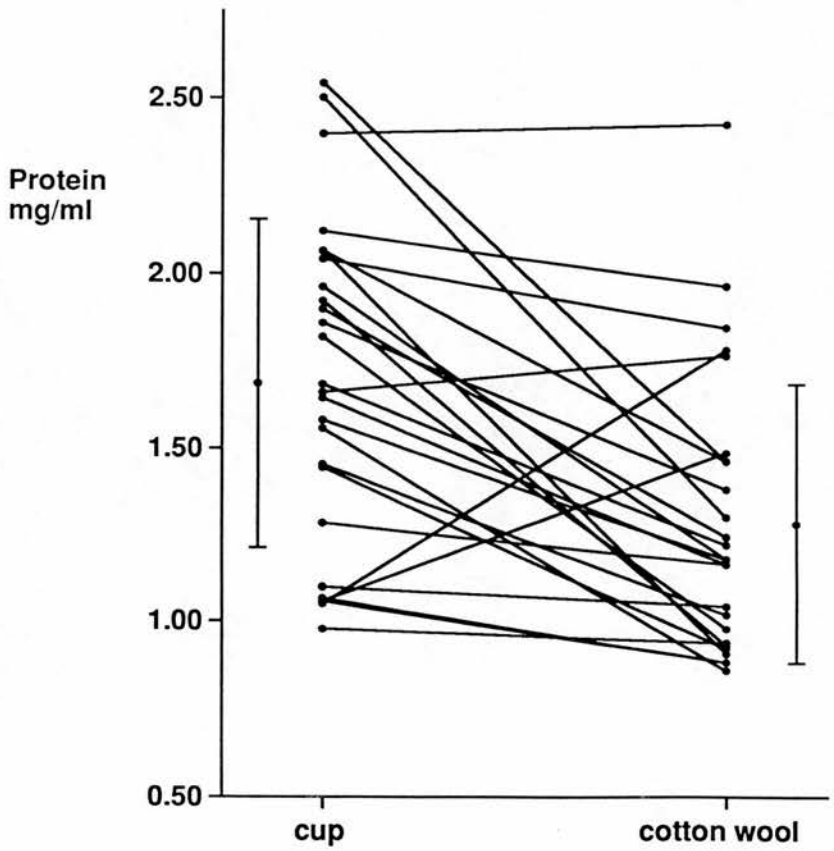
Carlsson-Crittenden cup was positioned over a parotid duct on one cheek. (This simple device has an outer ring with connected to an aspiration tube so that gentle suction may be applied to maintain cup position over the parotid duct orifice, and an inner collection cup, again connected to an aspiration tube through which the parotid saliva can be collected - see figures 4.1 and 4.2.

Figure 4.2 - Carlsson-Crittenden cup head in detail

A cotton-wool ball was positioned over the contralateral parotid duct as described above. Saliva collected by the cotton-wool was retrieved from the absorbent material by placing the material into the barrel of a 20 ml syringe, replacing the plunger and compressing the cotton-wool.

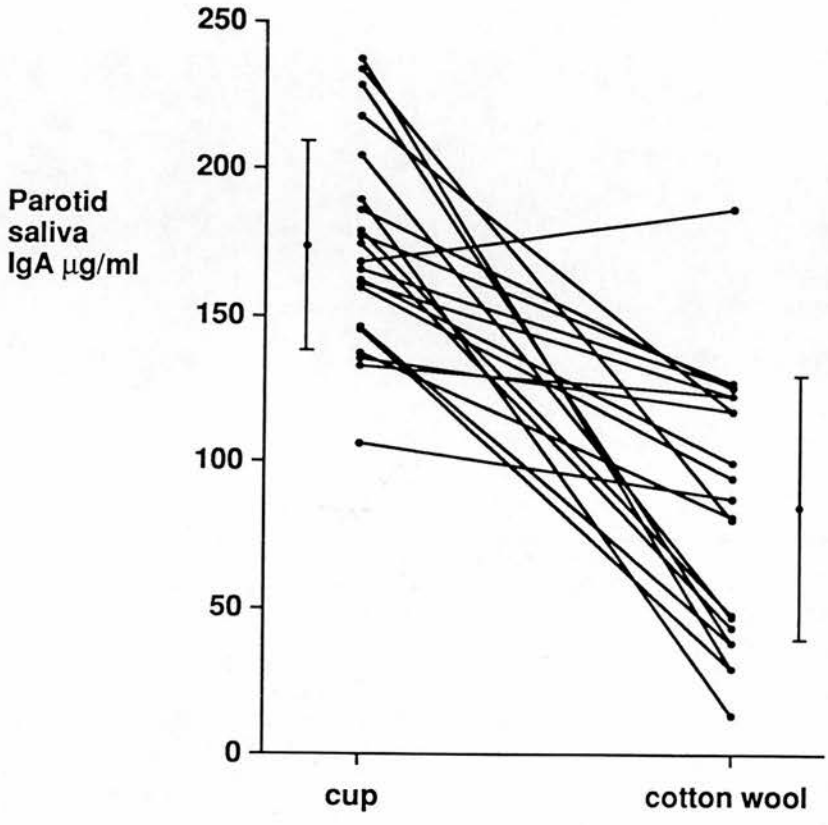
This forced absorbed saliva from the cotton-wool and out of the syringe. Parotid salivary samples were stored at -20^{oC} without any treatment or preservative until assay. Some minor problems were experienced with cup slippage away from the parotid orifice, especially in subjects with bulky masseter muscles, but generally collection was straightforward. Total protein and total IgA concentrations were assayed in the samples. Both total protein .

Figure 4.3 **Parotid salivary protein concentration**



Parotid salivary protein concentration when collected by Carlsson-Crittenden cup and cotton wool simultaneously. P=0.00001, (paired t-test).

Figure 4.4 **Parotid salivary IgA concentration**



Parotid salivary IgA concentration when collected by Carlsson-Crittenden cup and cotton wool simultaneously. P=0.00001, (paired t-test).

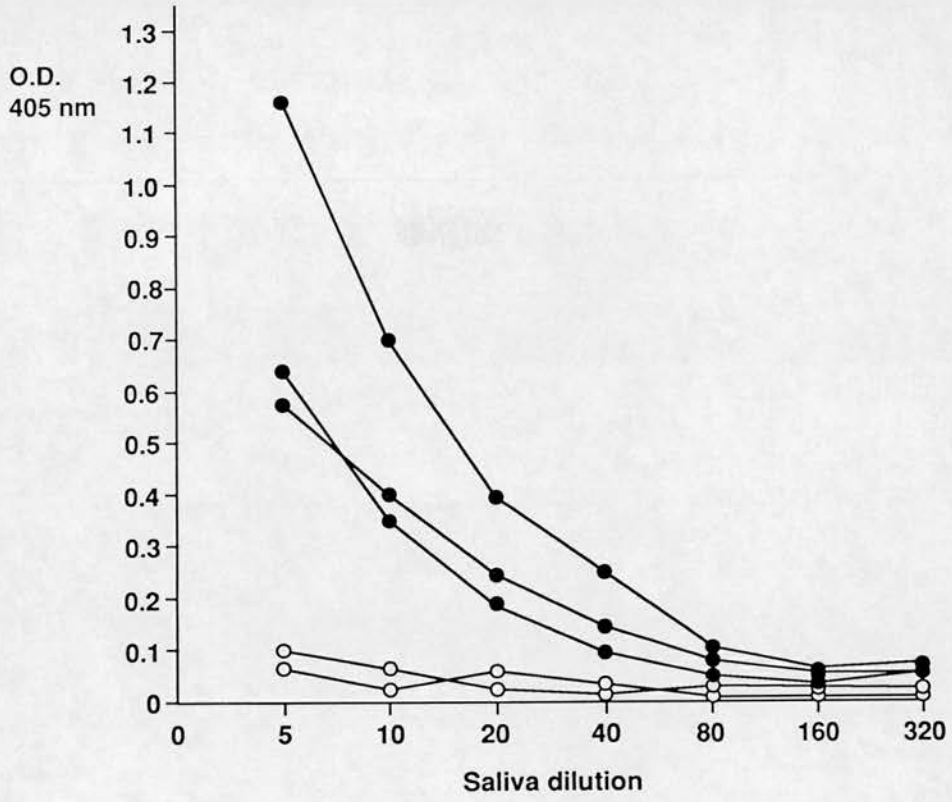
concentration (p=0.00001), shown in figure 4.3, and total IgA concentration (p=0.00001), shown in figure 4.4, were decreased in the parotid saliva collected by cotton-wool when compared with the saliva collected by cup.

Specificity of ELISA.

To ensure that the ELISA used was specific for immunoglobulins and antibodies in the parotid saliva,

standard serial dilution and absorption experiments were carried out. Serial dilution of the samples resulted in a parallel decline in activity (figure 4.5). Serial absorption of the antibodies also led to a stepwise and parallel reduction in activity (figure 4.6). Both of these experiments confirmed the specificity of the assay.

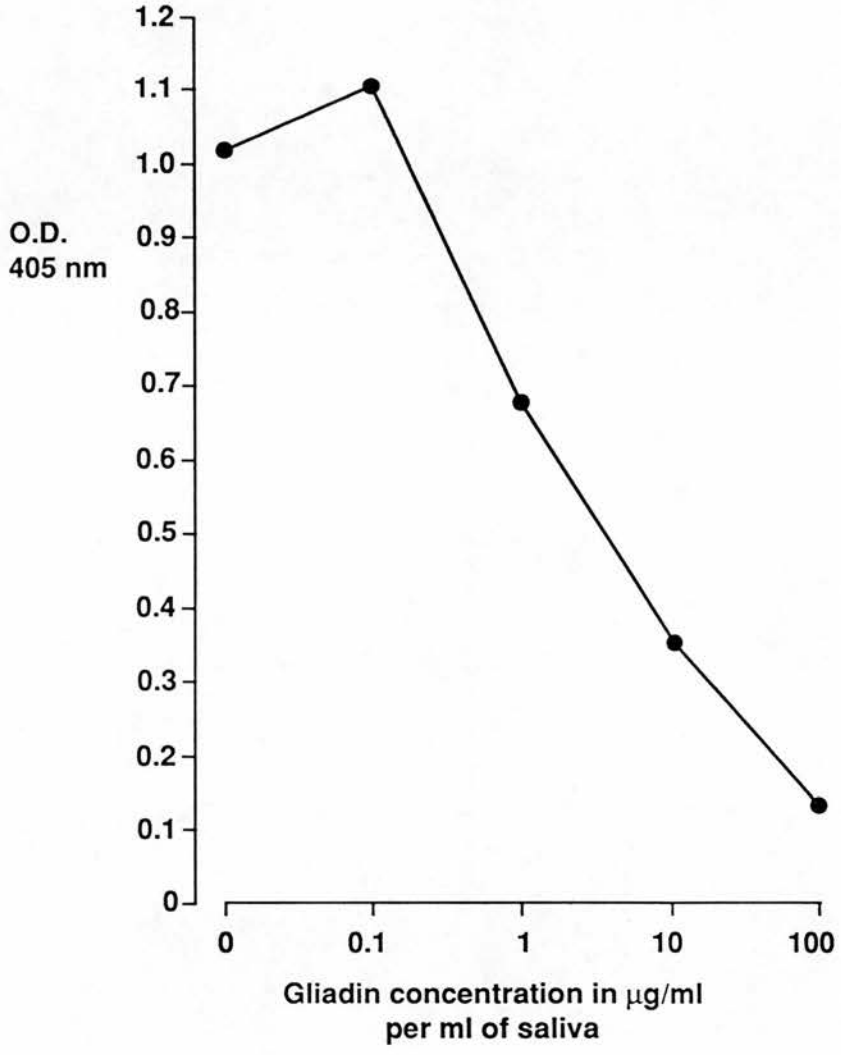
Figure 4.5 **ELISA specificity - serial dilutions**



Effect of serial dilution on optical density reading for IgA anti-gliadin antibody activity in samples of parotid saliva from five subjects.

Since small amounts of mucus are present in parotid saliva, and IgA has been shown to be associated with mucus, a variety of physical and chemical treatments were administered to unfiltered fresh parotid saliva in an attempt to increase measurable protein and IgA.

Figure 4.6 **ELISA specificity - absorption study**

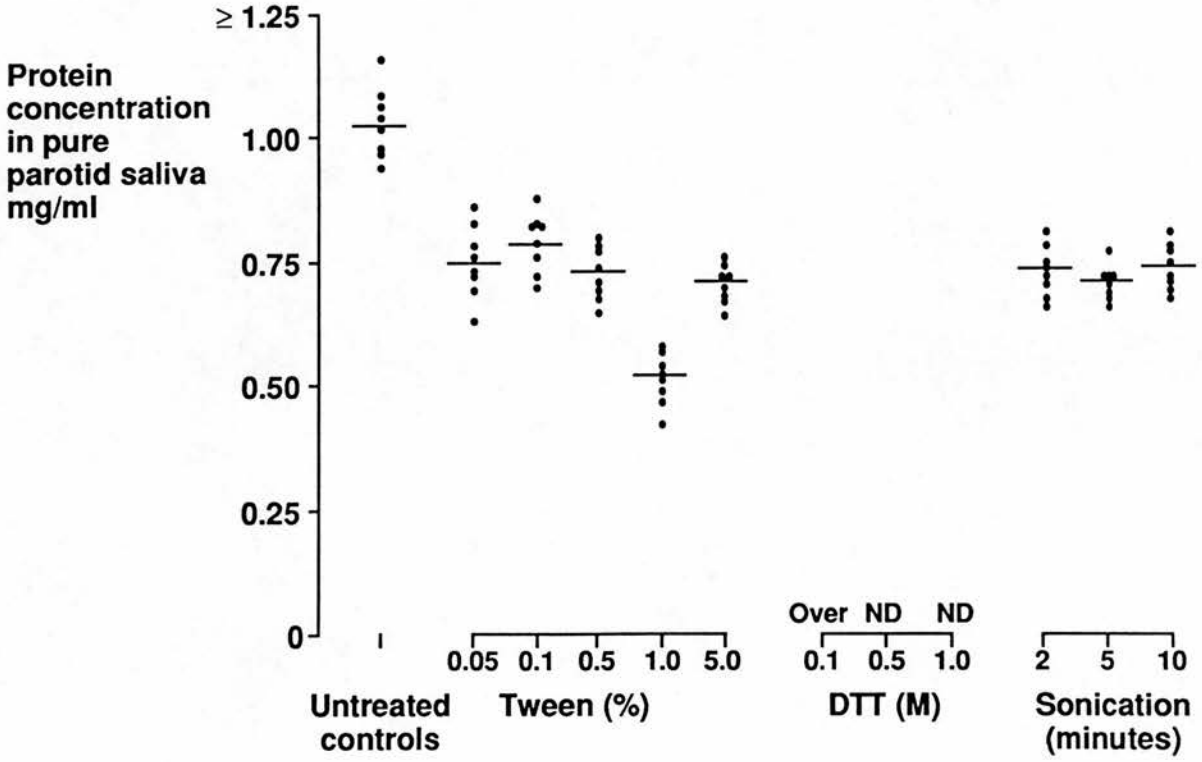


Optical density readings for IgA anti-gliadin antibody activity of parotid saliva after absorption against increasing concentrations of gliadin.

A range of concentrations of the detergent polyoxyethylenesorbitan (Tween, Sigma Chemical Co, Poole, Dorset), of the mucolytic dithiothreitol (DTT, Sigma Chemical Co.), and ultrasonification for different lengths of time, were used on the parotid saliva. The effect of filtration or

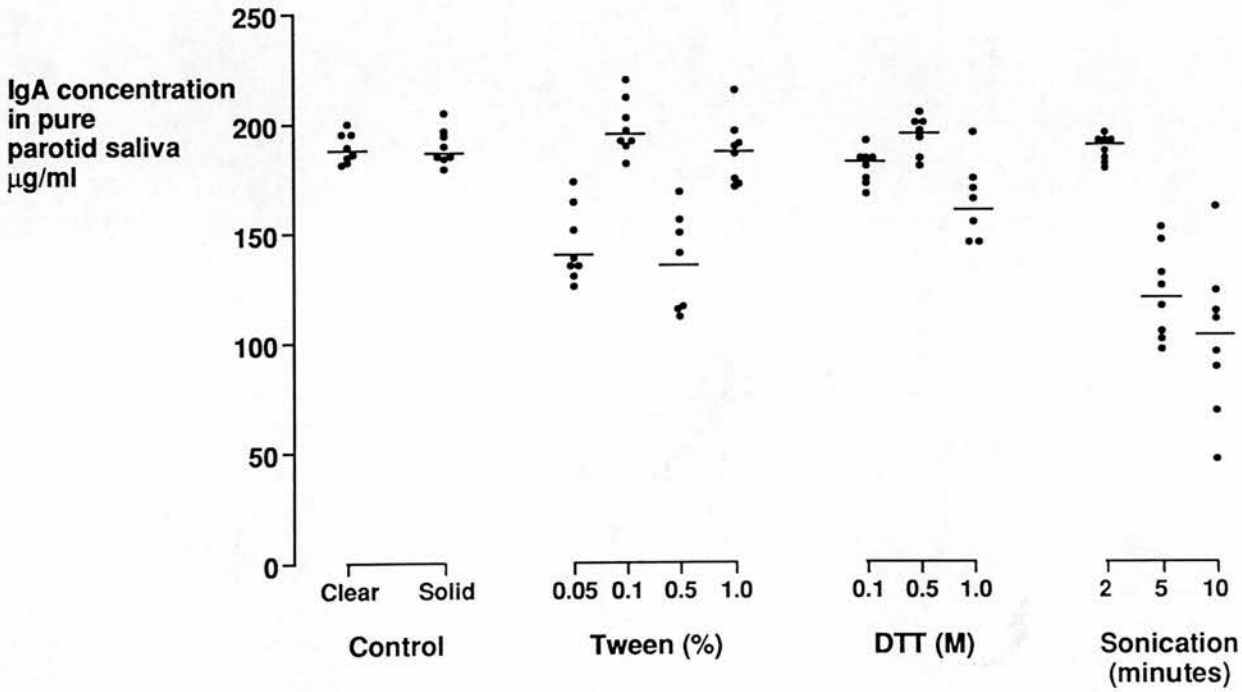
centrifugation, to remove any solid or mucus particles, on the measured protein and IgA was also assessed. No treatment increased measured total protein or IgA concentrations, but assay interference occurred with 0.1M DTT, resulting in unmeasurably high optical density (OD) readings (figures 4.7 and 4.8). Removal of mucus and solid particles by filtration or centrifugation did not alter measured IgA concentration (figure 4.8).

Figure 4.7 **Salivary protein concentrations after various treatments**



Measurable protein concentration after treating saliva with detergent (Tween), dithiothreitol (DTT), and sonication. Key- ND = not detected, over = reading above scale.

Figure 4.8 **Salivary IgA concentrations after various treatments**



Measurable IgA concentration after treatment of saliva by a detergent (Tween), dithiothreitol (DTT), and sonication. Saliva with (solid) and without mucus or solid particles (clear) was also compared.

Contamination

To assess if contamination of parotid saliva by other fluids could occur during the collection period, 5 mls of cochineal food colouring was pipetted into the oral cavity and a standard collection of saliva carried out. The salivary specimens were assessed by spectrophotometer for evidence of colouration, and compared with known serial dilutions of cochineal. Rinsing the mouth with

cochineal revealed no evidence of contamination of parotid saliva collected by Carlsson- Crittenden cup down to a dilution of $<10^{-5}$ on 10 occasions.

Assay reliability

The reproducibility and variation of ELISA analysis of serum and salivary total immunoglobulins and specific antibodies was assessed. For saliva IgA, IgM, and IgG immunoglobulin and antibodies were studied, and for serum IgA immunoglobulin and IgA isotype-specific antibodies were tested. For each immunoglobulin and antibody in both fluids, twelve specimens were assayed, each aliquoted into eight wells on a single ELISA plate to assess in-plate variation. For each of these eight samples the mean OD reading of each sample was determined and the range of OD readings expressed as a percentage variation from this mean. The results from the 12 samples were combined to give a mean percentage variation with a range.

In a separate experiment twelve samples were assayed in duplicate on three separate plates, and the mean OD reading for each sample determined on each plate. For each sample the mean variation between the plates was then calculated and expressed as a percentage. The percentage variations determined both within and between plates are shown in tables 4.1 and 4.2, and were within acceptable limits.

Table 4.1 **Within-plate variation of ELISA**

Specimen and assay details	Percentage deviation from average OD reading (mean + range)			
	total	anti-gli	anti-ova	anti-ßLG
Saliva - IgA	3.1 (1.9-4.4)	7.3 (4.2-8.9)	4.7 (3.2-7.0)	4.2 (2.3-6.4)
- IgG	4.0 (2.2-6.1)	8.7 (4.3-13.1)	5.3 (4.0-6.9)	4.6 (2.3-7.0)
- IgM	4.7 (2.5-6.6)	8.4 (5.7-9.9)	5.7 (4.0-7.6)	6.0 (3.9-8.4)
Serum - IgA	3.4 (2.0-4.7)	5.0 (2.8-8.3)	10.6 (5.9-16.9)	4.7 (2.5-6.4)

Twelve specimens of each isotype were aliquoted into 8 separate wells on the same plate for both saliva and serum.

Table 4.2 **Between-plate variation of ELISA**

Specimen and assay details	Percentage deviation from average OD reading (mean)			
	total	anti-gli	anti-ova	anti-ßLG
Saliva - IgA	5.8	11.1	8.4	6.9
- IgG	4.4	12.2	7.0	7.8
- IgM	6.9	9.1	9.4	9.1
Serum - IgA	5.2	7.1	13.4	5.5

Mean variation between three plates for 12 specimens tested in duplicate on each plate.

LAVAGE

Introduction

Patients attending the gastrointestinal unit who were about to undergo barium enema examination or colonoscopy, or who suffered from constipation, were offered bowel preparation with an alternative method of cleansing to the standard regime of sodium picosulphate and co-danthromer. The lavage solution employed for this purpose was a polyethyleneglycol (PEG) electrolyte lavage solution. PEG 3350, a mixture of different-sized molecules with a mean molecular weight between 3200 and 3700, and sulphate are the osmotic agents in the lavage solution. The solution is available commercially in the USA (and has now become available in the U.K. during the writing of this thesis) as "Golytely" and is used frequently and successfully as a method of cleansing the colon for colonoscopy, barium enema, and as a bowel preparation prior to abdominal surgery (Davis et al , 1980).

Formulation of lavage

The polyethylene glycol electrolyte lavage solution was formulated by the Hospital Pharmacy and contained Potassium chloride BP 0.75g, Sodium chloride BP 1.45g, Sodium bicarbonate BP 1.63g, Sodium bisulphate BP 5.68g and PEG 3350 59.1 g/L, with a resultant osmolality of approx. 260 mosmol/L.

Lavage protocol

Following an overnight fast, lavage was commenced at 8.30 am. Subjects were asked to drink the lavage solution at a rate of 250 mls every 15 minutes for a period of 4 hours, making the total volume consumed 4 litres. In patients in whom lavage was used as a bowel preparation, one sachet of sodium picosulphate ("Picolax"), was given later the same day to remove excess fluid from the colon which might interfere with radiographic studies.

Specimen processing

Since human intestinal secretions contain large amounts of proteases, the specimens were treated with protease inhibitors to preserve their immunoglobulin content. The method of specimen processing described was adapted from that developed by Elson and colleagues for use in the mouse (Elson et al 1984). Stool collection was commenced once the stools became liquid and free of faecal material. Approximately 200 mls were collected and filtered through cheesecloth into 50 ml polypropylene tubes. Soybean trypsin inhibitor (Sigma Chemical Co.) 1mg/ml in phosphate-buffered saline (PBS) was added to each tube to a final concentration of 10% (W/V), followed by addition of 1.0m ethylene-diamine-tetracetic acid (EDTA) to 5% (W/V) in PBS. Tubes were then centrifuged at 550 g for 10 minutes.

The supernatant was transferred into clean centrifuge tubes and 1% 100 mM phenylmethylsulfonylfluoride (PMSF) in 95% alcohol was added to 1% (W/V). Tubes were then

centrifuged at 2200 g for 30 minutes. The supernatant was taken and PMSF was again added to a total of 1% (W/V), followed by the addition of 1% (W/V) Sodium azide in PBS. After 15 minutes gentle mixing, 5% (W/V) heat-inactivated fetal calf-serum was added. Samples were divided into aliquots and stored at -70^{0C} until assay. In 10 subjects, the initial sample was divided into 3 aliquots, one being treated immediately, and the other addition of protease inhibitors delayed for 1 and 2 hours, respectively, in the other 2. In 14 subjects, 2 serial specimens were collected and analysed for IgA content.

Measurement of PEG levels in lavage samples

PEG was quantified by the method of Malawer (Malawer and Powell, 1967), a modification of the turbidimetric analysis of Hyden (Hyden, 1955).

Results

Subject acceptance

Of 78 subjects who began lavage, 75 subjects were able to provide samples suitable for analysis. Two subjects could not tolerate the procedure, and one specimen was found to be mainly urine on analysis. Subject acceptance was reasonable; the solution was more acceptable if chilled and taken with lemon juice. In some patients who did not

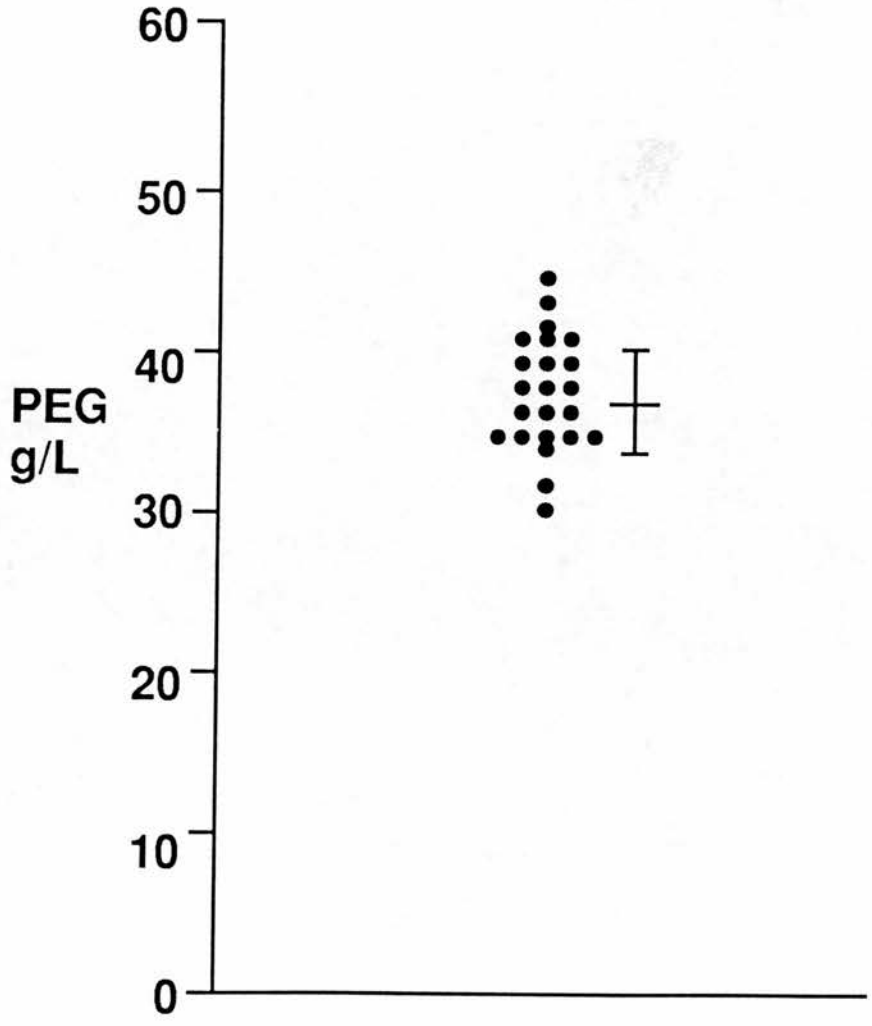
tolerate the lavage, in whom it was necessary to complete the preparation fully, a naso-gastric tube was passed to administer the solution. The only side-effects were abdominal bloating and nausea. Occasionally vomiting of the fluid led to abandonment of the lavage, but generally it also could be easily accomplished in an out-patient setting. A medical presence was generally not required, supervision being conducted at a distance. The gastrointestinal unit investigation suite nursing staff became extremely adept at administering the lavage fluid to subjects and only occasionally was it necessary to introduce a naso- gastric tube for that purpose.

Immunoglobulin content

The time from the start of lavage to the passage of clear stool specimens suitable for analysis ranged from 1 - 5 hours. Assay of the PEG showed little variation in concentration (figure 4.9). Immunoglobulins and antibodies were barely detectable if solid faecal material was still present at the time of specimen collection, and it seemed necessary to ensure that faecal effluent was clear before samples were obtained. Initially, all stools were collected, until assay of these serial specimens demonstrated that, once a clear effluent was produced, there were no further significant changes in IgA concentrations, suggesting that a steady state had been reached (figure

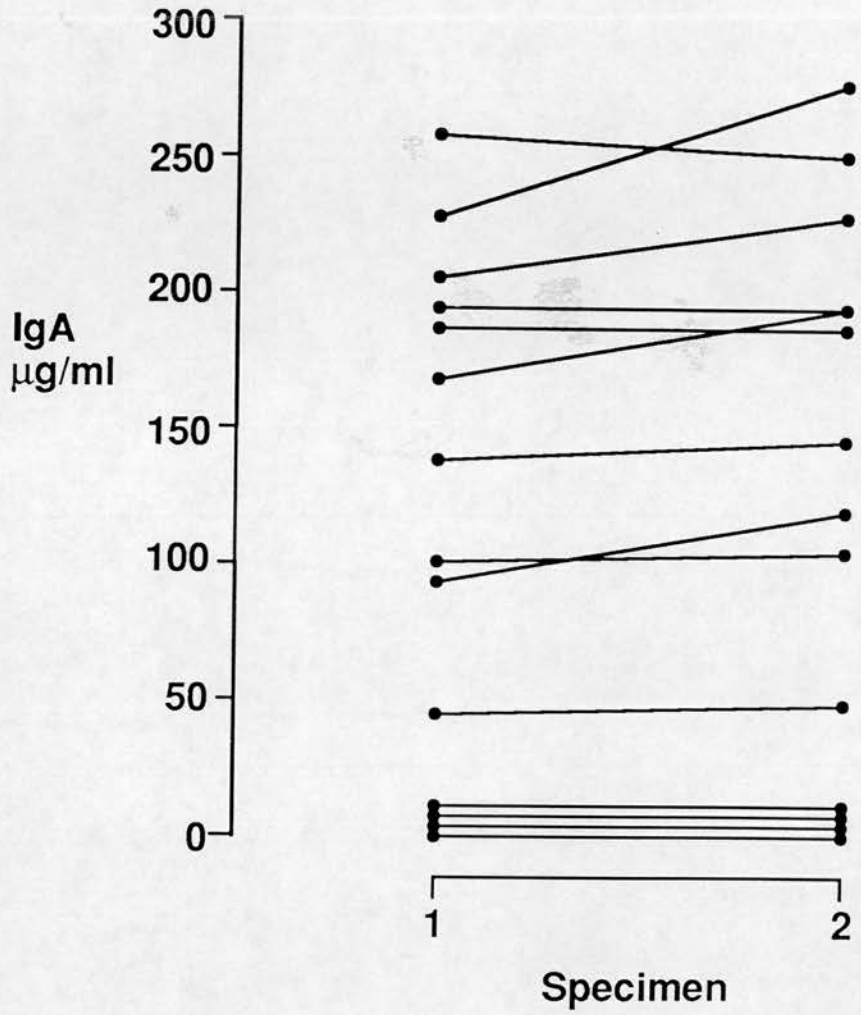
4.10), and therefore that sample timing was not crucial. Delay in the addition of protease inhibitors of one or two hours resulted in a variable (0-92%) loss of IgA activity (figure 4.11). This loss of immunoglobulin content due to delay in the addition of protease inhibitors has been noted by others, who also found that the

Figure 4.9 **PEG concentration in lavage fluid**



Variation in PEG concentration in lavage samples from 23 subjects. Mean concentration was 37.3 g/L (sd ±3.4).

Figure 4.10 **IgA concentration in lavage**

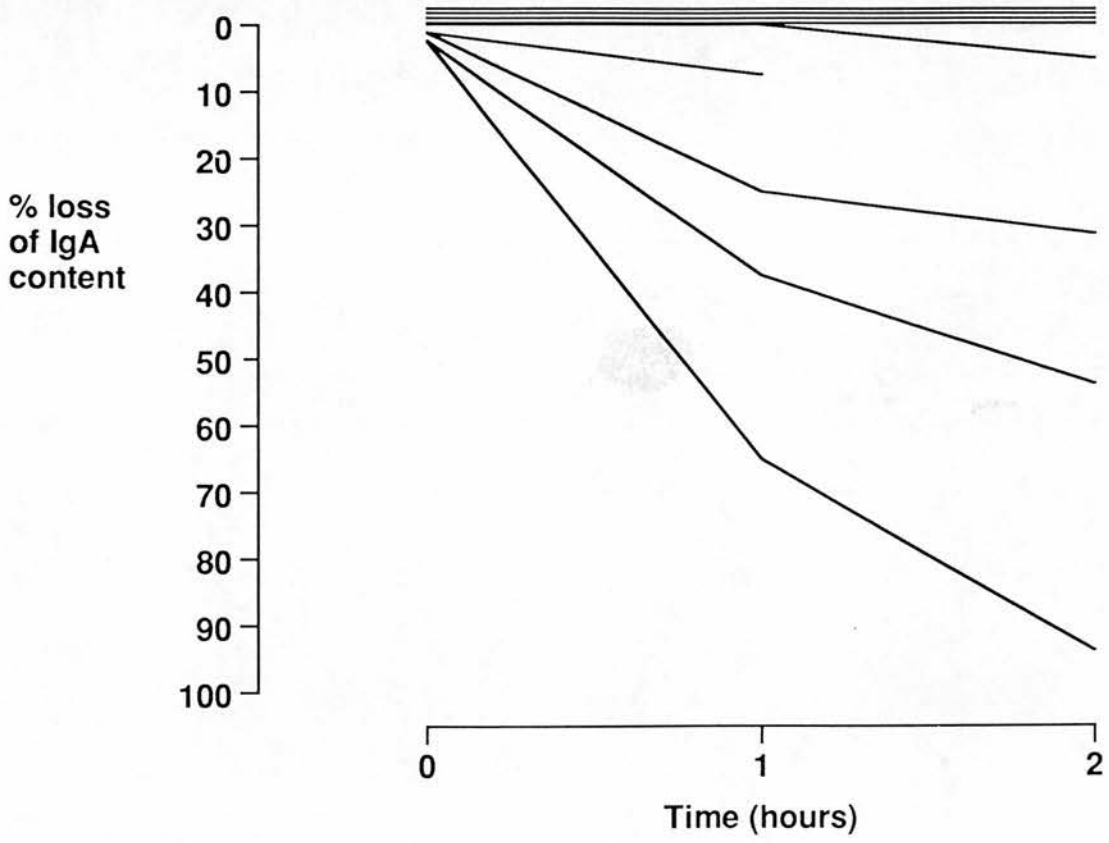


IgA concentration in two consecutive lavage samples in 14 subjects, after effluent became clear.

inhibitors did not interfere with assay by ELISA, which was sensitive and showed minimal variation (Gaspari et al, 1988). The initial PEG concentration of 59 g/L in the ingested fluid would be reduced to 47.5 g/L by the addition of protease inhibitors etc. Since the percentage of

gut secretion in the liquid faecal effluent is determined by a simple formula, (100 - the sample PEG concentration [g/L] ÷ 47.5 [g/L]), the samples were therefore a mean 78.5% PEG-electrolyte solution and 21.5% gut secretions.

Figure 4.11 **Effect of delay in addition of protease inhibitors to IgA concentrations in lavage fluid.**



Comments

The choice of PEG-electrolyte solution as the lavage fluid was straightforward. With minimal net water and electrolyte absorption or secretion, potential problems of fluid overload in susceptible patients are avoided (Davis et al, 1980), although others have suspected some fluid absorption as reflected by increased urinary volumes (DiPiro et al, 1986). In a small colonic perfusion

study, greater than 97.3% of PEG from healthy volunteers, and more than 96.5% of PEG from patients, was recovered from the gut (Shields et al, 1968), and only 0.04-0.06% of a total PEG dose administered orally was recovered in urine (DiPiro et al, 1986). Previously reported side-effects of mild nausea and abdominal distension or discomfort (DiPiro et al, 1986; Gaspari et al, 1988) were also encountered in this study, but although a small number of subjects were unable to retain the lavage solution, the majority tolerated it well.

ELISA protocols

After the dilution-ranging studies, all other studies of specificity and reproducibility were performed for each assay respectively, using the protocols specified in appendices (v)-(vii).

ELISA standards

For all ELISA's of total immunoglobulin concentrations, a colostral IgA standard was used (SPS 101; Human Protein Reference Unit, Royal Hallamshire Hospital, Sheffield), double diluted out as detailed in the appendices.

CHAPTER FIVE: THE PHYSIOLOGY OF SALIVARY IMMUNOGLOBULINS AND ANTIBODIES TO FOOD PROTEIN ANTIGENS

Introduction

In order to ensure that the results obtained from salivary samples were relevant and representative it was necessary to answer a number of questions. The aims at this stage were to determine if parotid salivary immunoglobulin and antibody levels varied significantly throughout the day. This was necessary to determine whether or not a random salivary sample, taken for example in an out-patient clinic, was representative of salivary immunity throughout the 24 hour period. Secondly, it was necessary to determine whether or not total protein concentration and salivary flow rate had significant influence on salivary immunity and would therefore be important to measure. Thirdly, the influence of dietary antigen intake and alcohol consumption were assessed in order to determine if these factors would need to be taken into account when interpreting results.

Approach and results

Circadian rhythms.

Eight healthy adult volunteers were studied over a sixteen hour period in a single day, with parotid salivary samples taken at intervals throughout that time. The first sample was taken after an overnight fast, the second after a standard breakfast and so on, and the last sample prior to midnight. Samples were assayed for total

IgA, IgM, and IgG, and for IgA antibodies to gliadin. The variation in these samples was assessed statistically using the Kruskal-Wallis test. There were significant fluctuations in both total immunoglobulin concentrations and in IgA anti-gliadin antibody levels throughout the day ($p<0.0025$ IgA; $p<0.005$ IgM; $p<0.00025$ IgG; $p<0.001$ IgA antigliadin antibody), figures 5.1 and 5.2. The most consistent change was seen after meals, with a fall in immunoglobulin concentrations and antibody level.

Influence of eating.

In a further, separate, experiment specifically aimed at studying the effects of a meal, paired salivary samples were obtained after an overnight fast and 30 minutes later, after a standard breakfast. Samples were assayed for IgA concentration (n=11), and total protein concentration (n=13). These experiments confirmed a fall in IgA concentration ($p=0.0023$), figure 5.3, and showed a non- significant increase in total protein concentration ($p=0.075$), figure 5.4 (paired t-test). Pure parotid sali vary samples from 92 patients without gastrointestinal disease were studied to determine the correlations between total IgA, IgM, and IgG, total protein concentration, and salivary flow rate. IgM concentration was weakly but positively correlated with total protein concentration (figure 5.5). There were no other significant correlations (table 5.1).

Alcohol

A serendipitous observation on a healthy volunteer suggested that alcohol ingestion might result in profound changes in salivary antibody levels. Eight young adult volunteers were studied to clarify this. Each subject abstained from alcohol for five days, following which they ingested 180 grams of alcohol taken as 440 mls of gin, diluted freely with tonic water, over a six hour period. After the alcohol challenge, subjects abstained from alcohol for a further five days. Samples of pure parotid saliva were obtained on days -2, -1, and days +1 and +2, and a cellobiose-mannitol small intestinal permeability test performed on days 0 and +1. Salivary samples were assayed for total IgA, IgM, and IgG, and for antibodies to gliadin, ovalbumin, and ßlactoglobulin of all the three immunoglobulin classes. Random fluctuations in antibody levels were seen, wider than observed in the sixteen hour study of the variation of antibody levels in healthy subjects. No consistent effects were noted however. The cellobiose-mannitol ratio increased in one of the eight volunteers, suggesting an increase in gut permeability.

Influence of dietary antigens.

Pure parotid saliva and serum were obtained from 34 patients in whom the final diagnosis was of functional bowel disease. Samples were tested for antibodies to gliadin, ovalbumin, and ßlactoglobulin of IgA, IgM, and IgG classes. Comparisons of antibody levels were made

between the patients who included milk or eggs or bread in their diet and patients who excluded one or other of these foods from their diet.

There were no significant differences in antibody levels, either in saliva or serum, (figures 5.6-5.10, table 5.2). Although data on dietary antigen intake is likely to be fairly accurate for eggs (for ovalbumin), and for milk (ßlactoglobulin), this is probably not so for gliadin since it is found not just in bread but in all foods that contain flour, of which there are many. Thus it is likely that subjects who did not eat bread still consumed gliadin, although they may have taken quantitatively less than those who did include bread in their diets. Because of this, data for anti-gliadin antibodies in serum has not been analysed, and the data for anti-gliadin antibodies in saliva should be interpreted with caution.

Dietary antigen withdrawal.

To study the effects of withdrawing dietary antigens, serial parotid salivary samples were collected from eleven heterogeneous patients who were started on an elemental diet for clinical reasons. Samples were obtained prior to and during feeding solely with a commercially available, chemically defined, diet (Elemental 028, Scientific Hospital Supplies, Liverpool, U.K.). The patients were being variously treated for coeliac disease, Crohn's disease, or short bowel syndrome, and the duration of the elemental diet ranged from 7 to 34

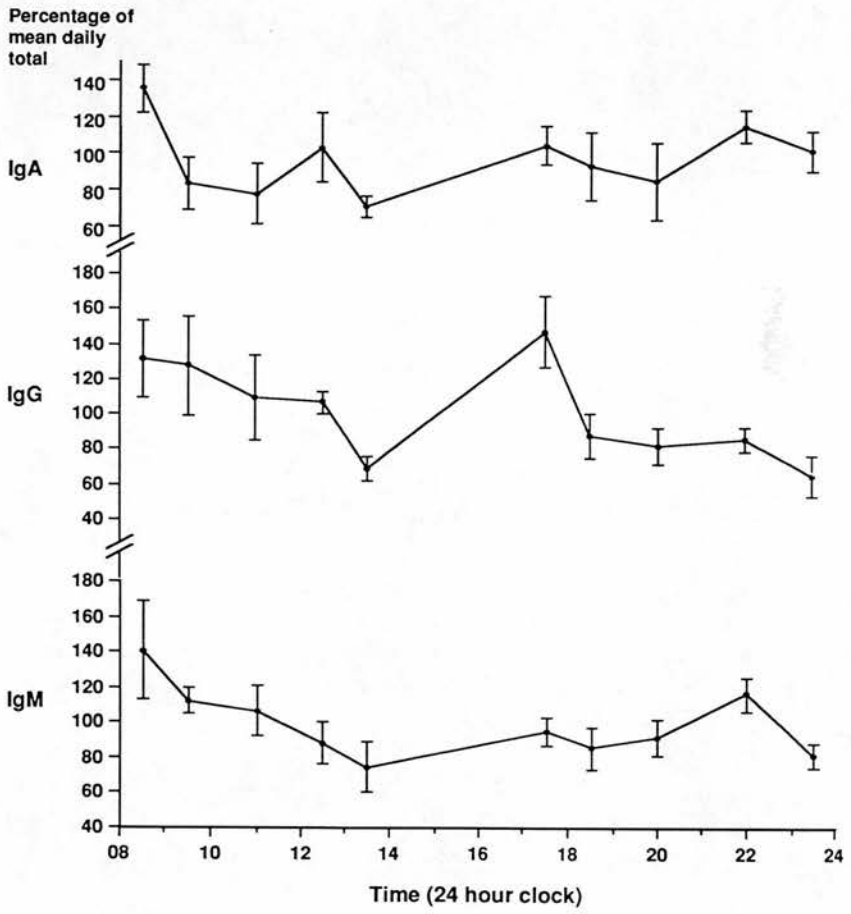
days.

Although antibody levels fell markedly in two patients, there was no significant change overall, for example IgA anti-gliadin antibody (figure 5.11). The results may reflect the heterogeneity of both disease groups and duration of study; also, other factors may need to be taken account of, additional medical therapy for example.

Conclusions

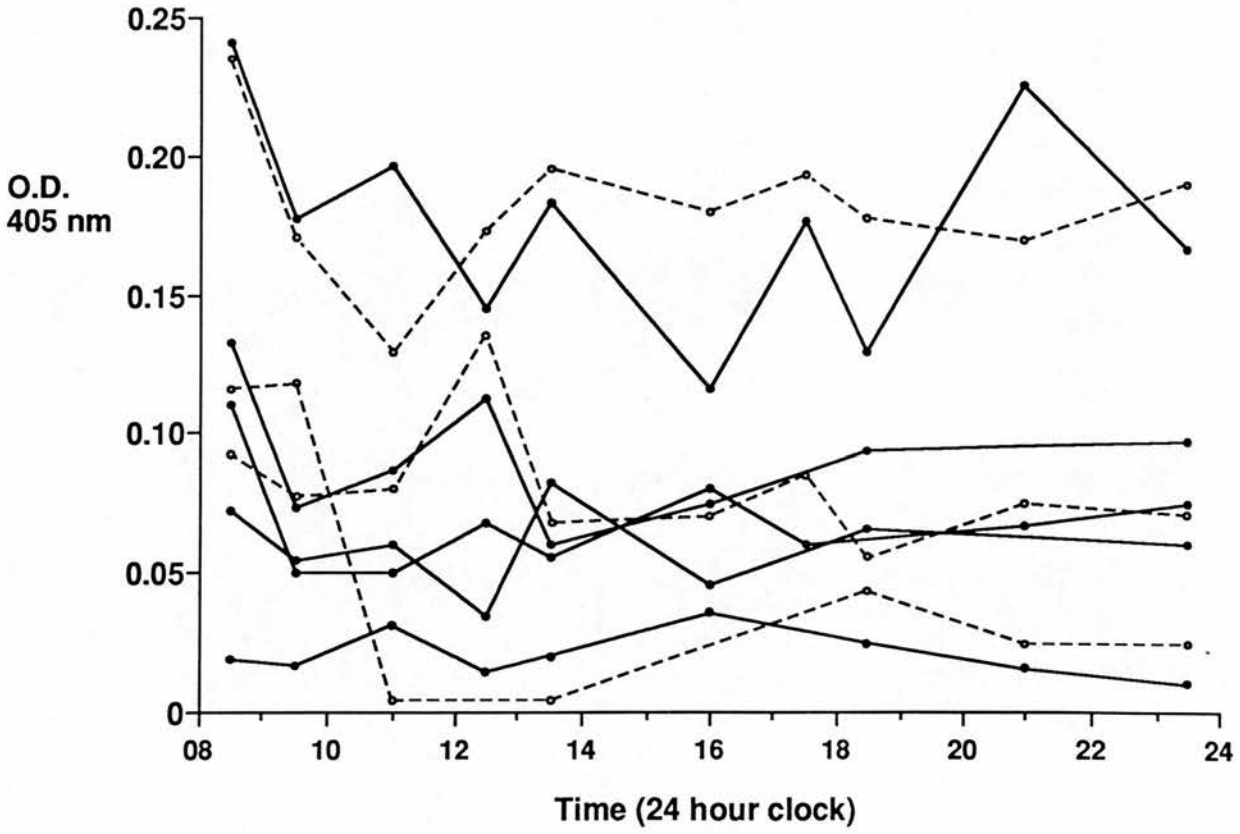
There is great physiological variation in immunoglobulin concentrations and antibody levels in pure parotid saliva during the day, and generally speaking, eating a meal reduces these parameters, especially after an overnight fast. Neither alcohol, nor the amount of an antigen in the diet appear to have any consistent effects on immunoglobulin concentrations or antibody levels in saliva. It seems reasonable in further studies on pure parotid saliva to collect saliva after fasting, accepting high individual variation, and allowing for the only definite influence of eating meals. It does not seem necessary to measure either salivary flow rate or total protein concentration.

Figure 5.1 **Daily variation of total immunoglobulin concentrations in parotid saliva.**



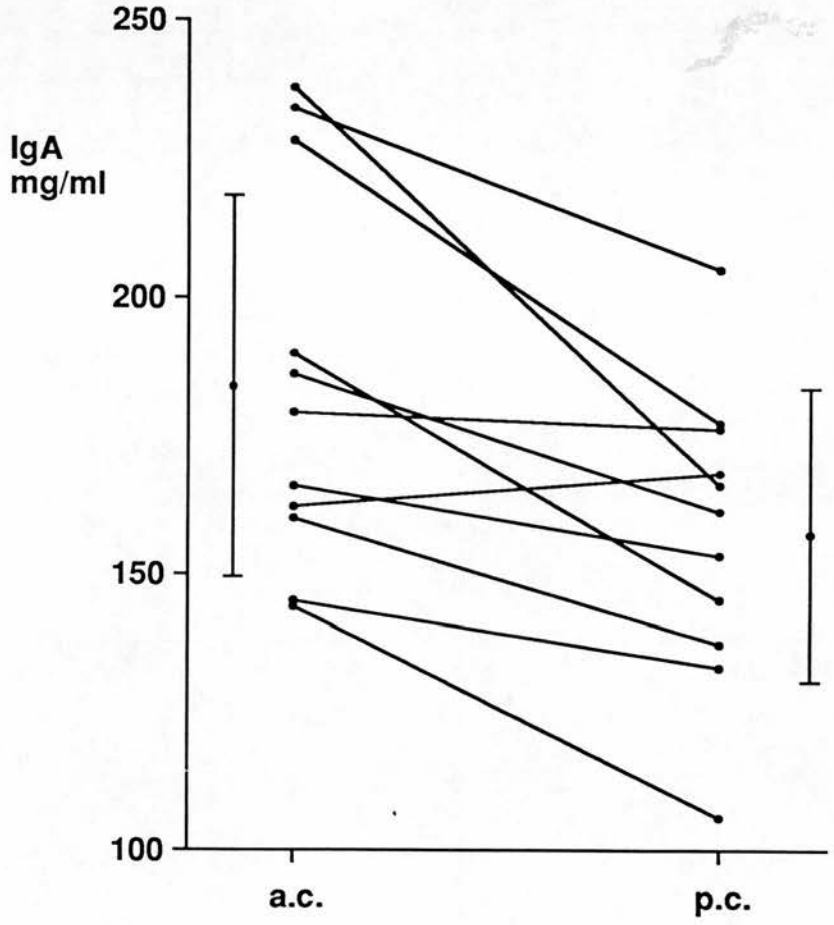
Each value expressed as a percentage of the mean daily total for that individual. All immunoglobulins vary significantly throughout the 24 hours, $p<0.005$, (Kruskal-Wallis test).

Figure 5.2 **Fluctuation in salivary IgA antigliadin antibody levels.**



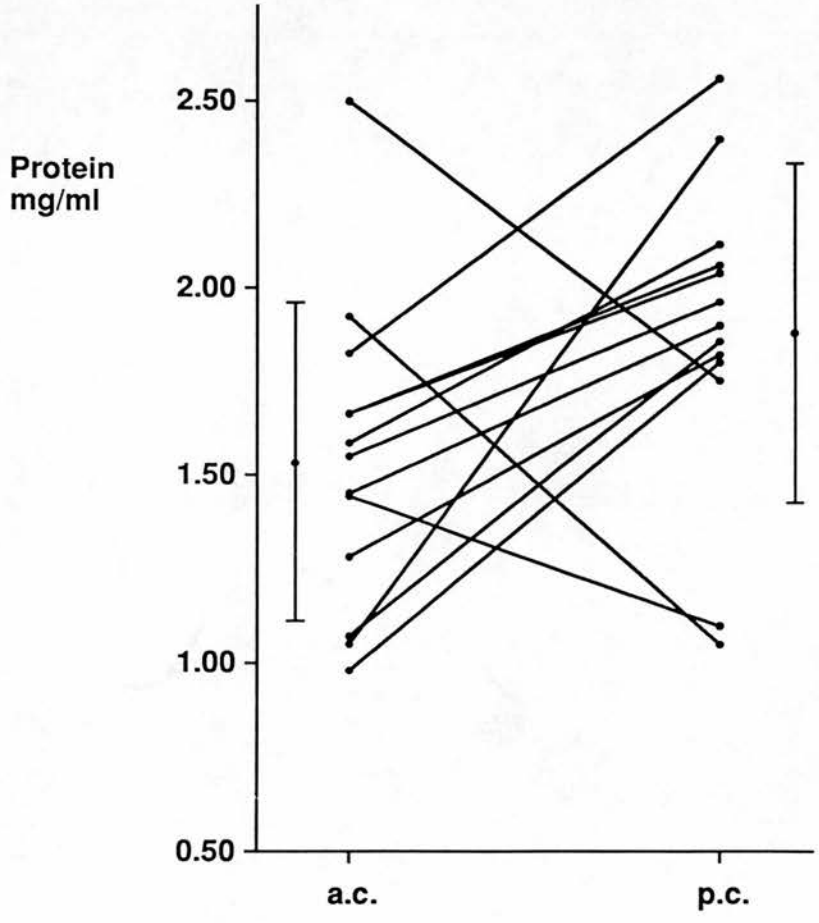
Fluctuation in salivary IgA antigliadin antibody levels over 16 hours in healthy subjects, (n=8). Alterations were significant, $p<0.001$ (Kruskal-Wallis test).

Figure 5.3 **Changes in IgA concentration in pure parotid saliva after a meal.**



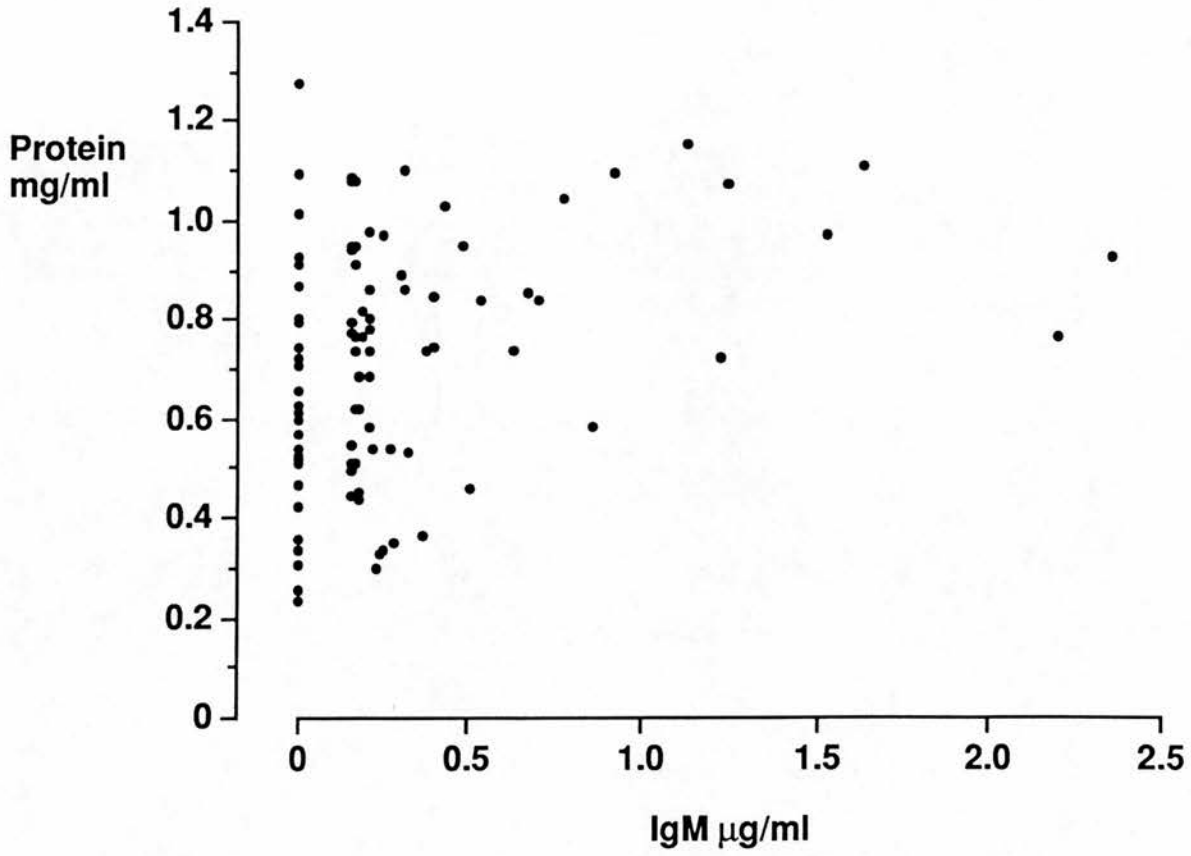
Changes in IgA concentration in pure parotid saliva, fasting (a.c.) and 30 minutes after a standard breakfast (p.c.), p = 0.0023 (paired t-test).

Figure 5.4 **Changes in total protein concentration in pure parotid saliva after a meal.**



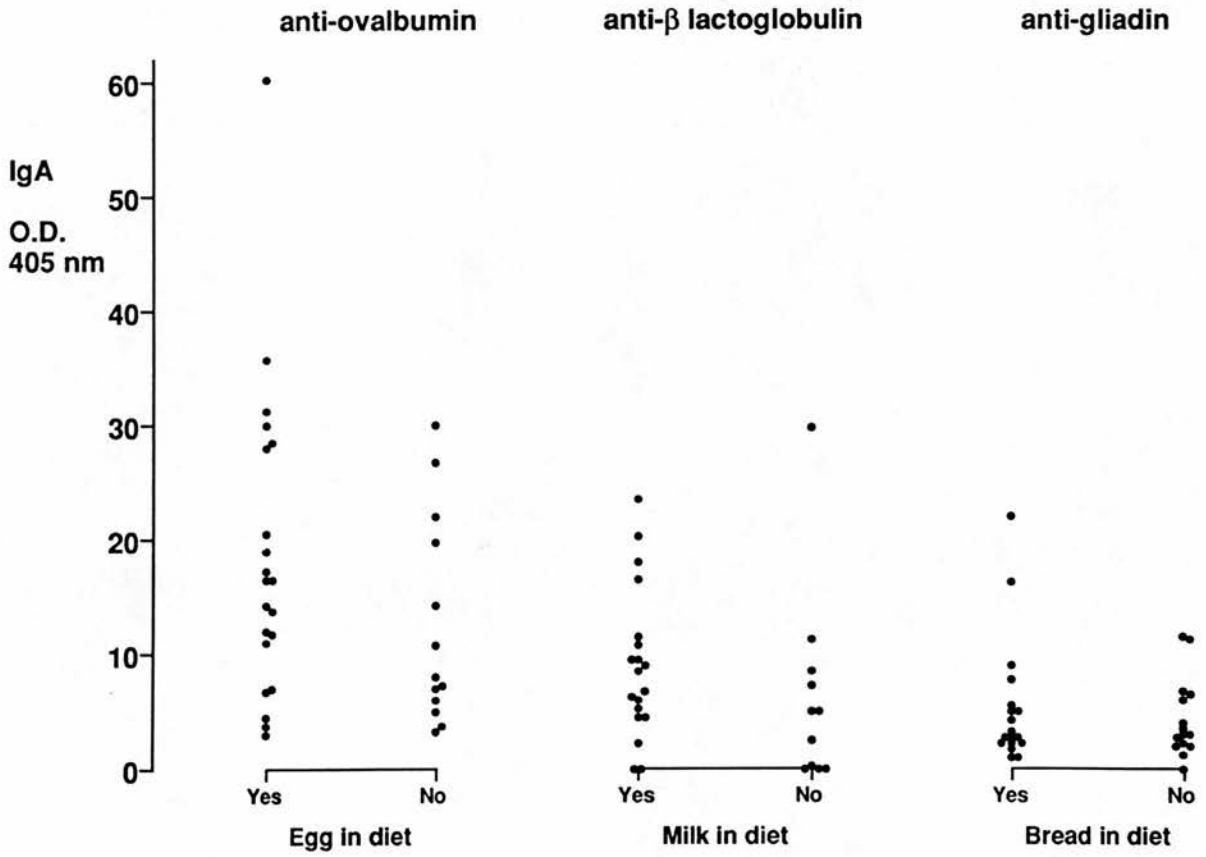
Changes in total protein concentration in pure parotid saliva, fasting (a.c.) and 30 minutes after a standard breakfast (p.c.), p = 0.075, (paired t-test).

Figure 5.5 **Correlation between total protein and IgM in pure parotid saliva.**



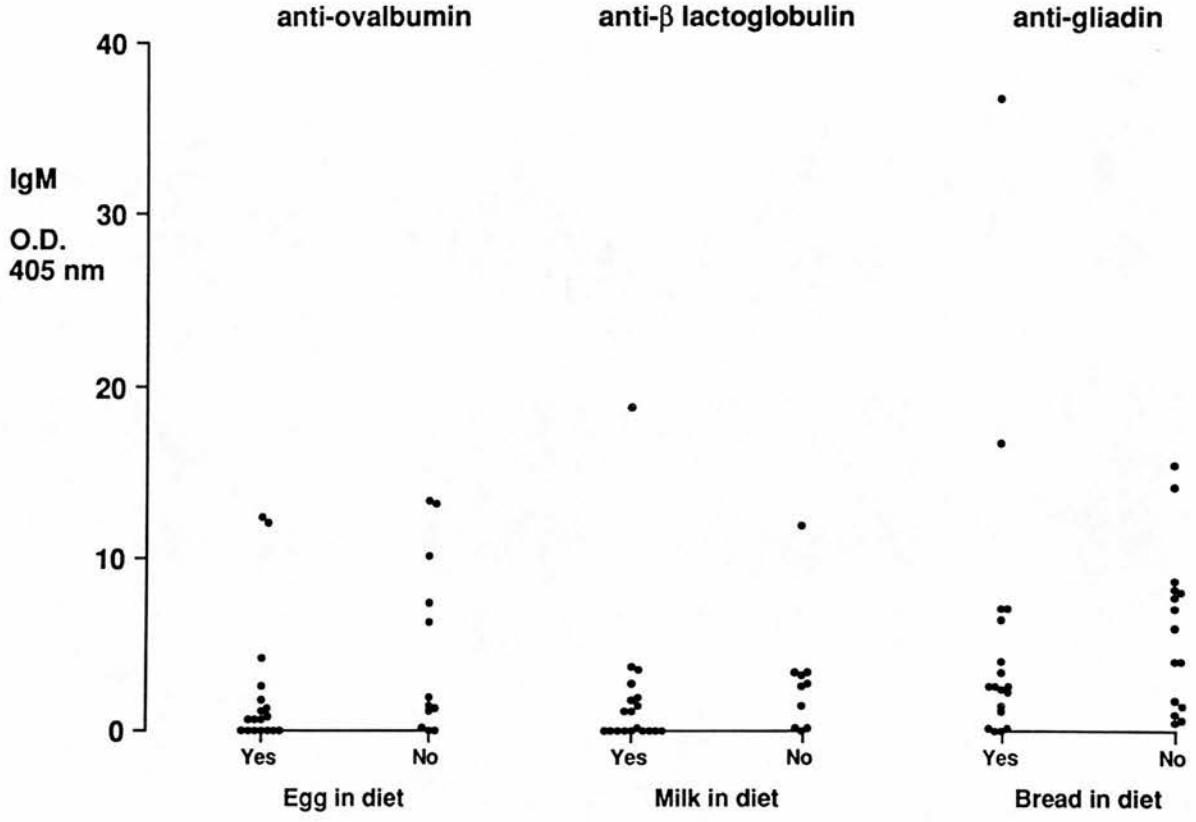
Relationship between total protein content and IgM concentration in pure parotid saliva; n = 92, correlation (r_s) = 0.276, p = 0.008.

Figure 5.6 **Dietary egg, bread, and milk, and specific salivary IgA antibody levels.**



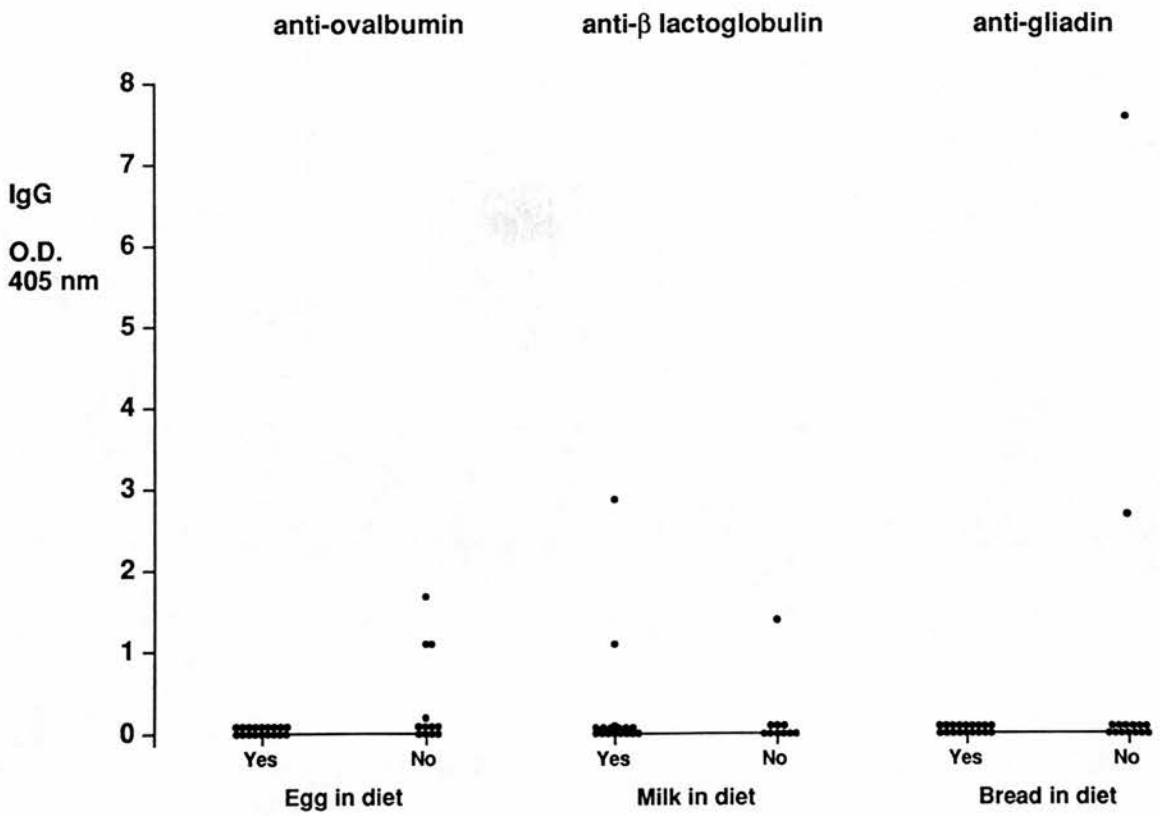
IgA antibody levels (OD readings) to food protein antigens in pure parotid saliva, comparing subjects omitting food with those including food in diet. Caution in interpreting data on anti-gliadin antibodies - see text. No significant differences (Mann-Whitney test).

Figure 5.7 **Dietary egg, bread, and milk, and specific salivary IgM antibody levels.**



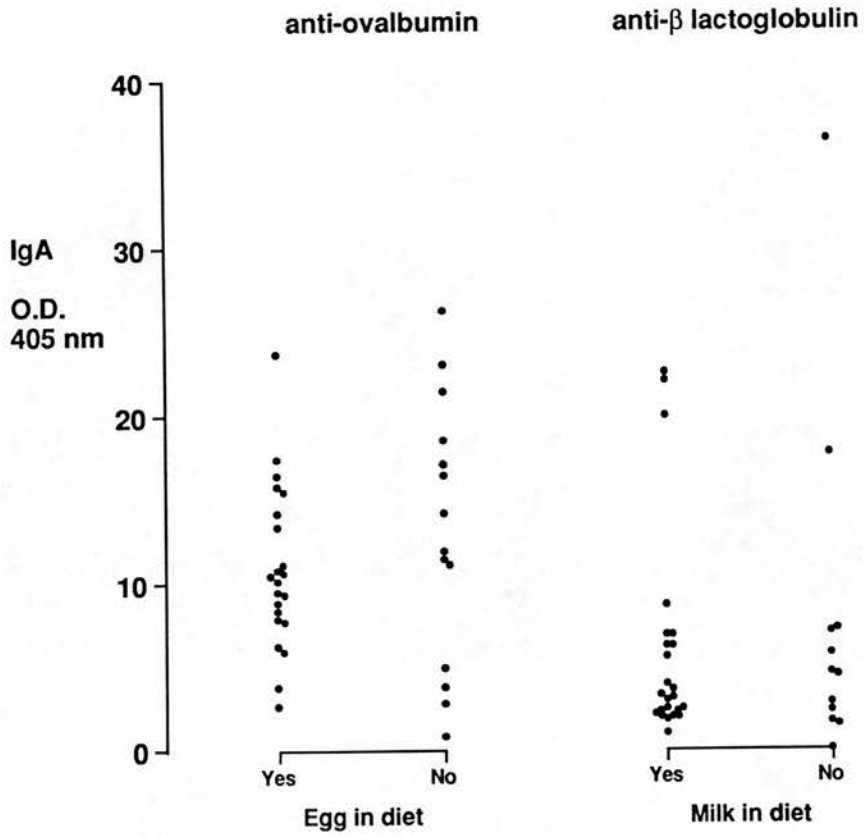
IgM antibody levels (OD readings) to food protein antigens in pure parotid saliva, comparing subjects omitting food with those including food in diet. Caution in interpreting data on anti-gliadin antibodies - see text. No significant differences (Mann-Whitney test).

Figure 5.8 **Dietary egg, bread, and milk, and specific salivary IgG antibody levels.**



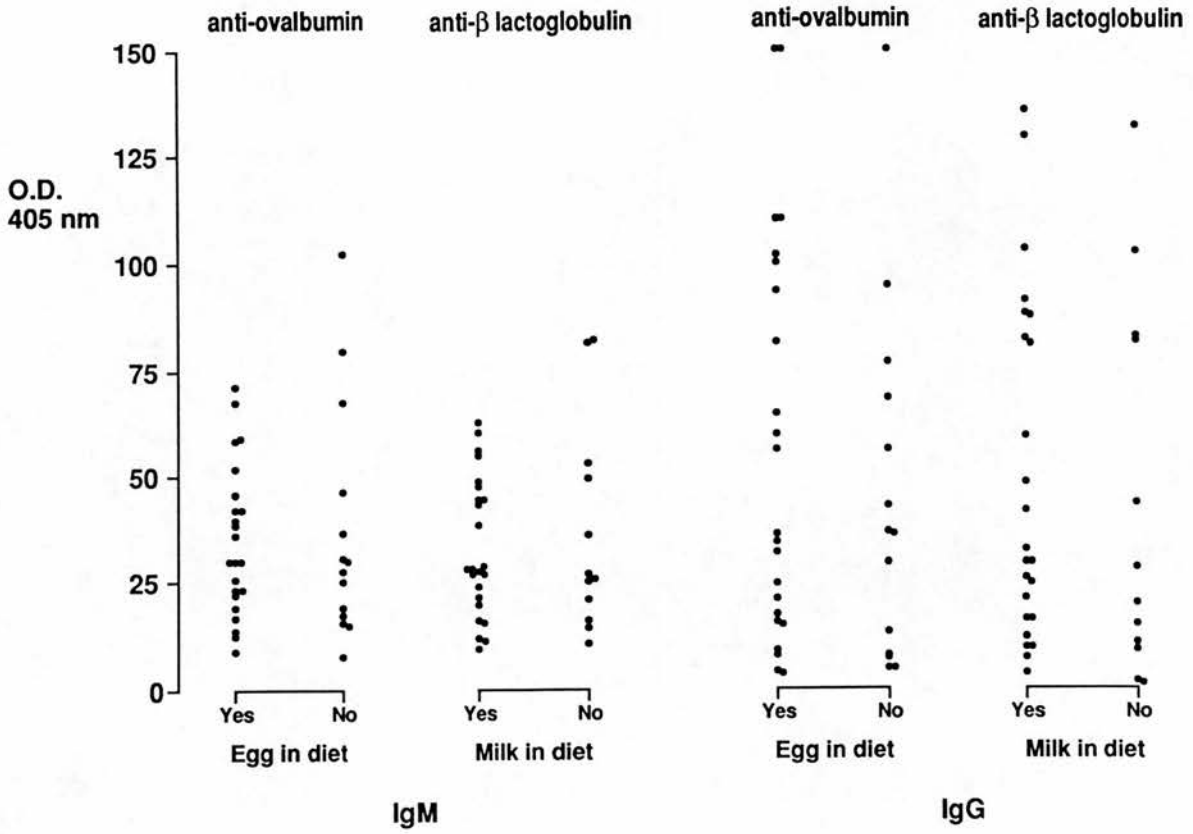
IgG antibody levels (OD readings) to food protein antigens in pure parotid saliva, comparing subjects omitting food with those including food in diet. Caution in interpreting data on anti-gliadin antibodies - see text. No significant differences (Mann-Whitney test).

Figure 5.9 **Dietary egg, bread, and milk, and specific serum IgA antibody levels.**



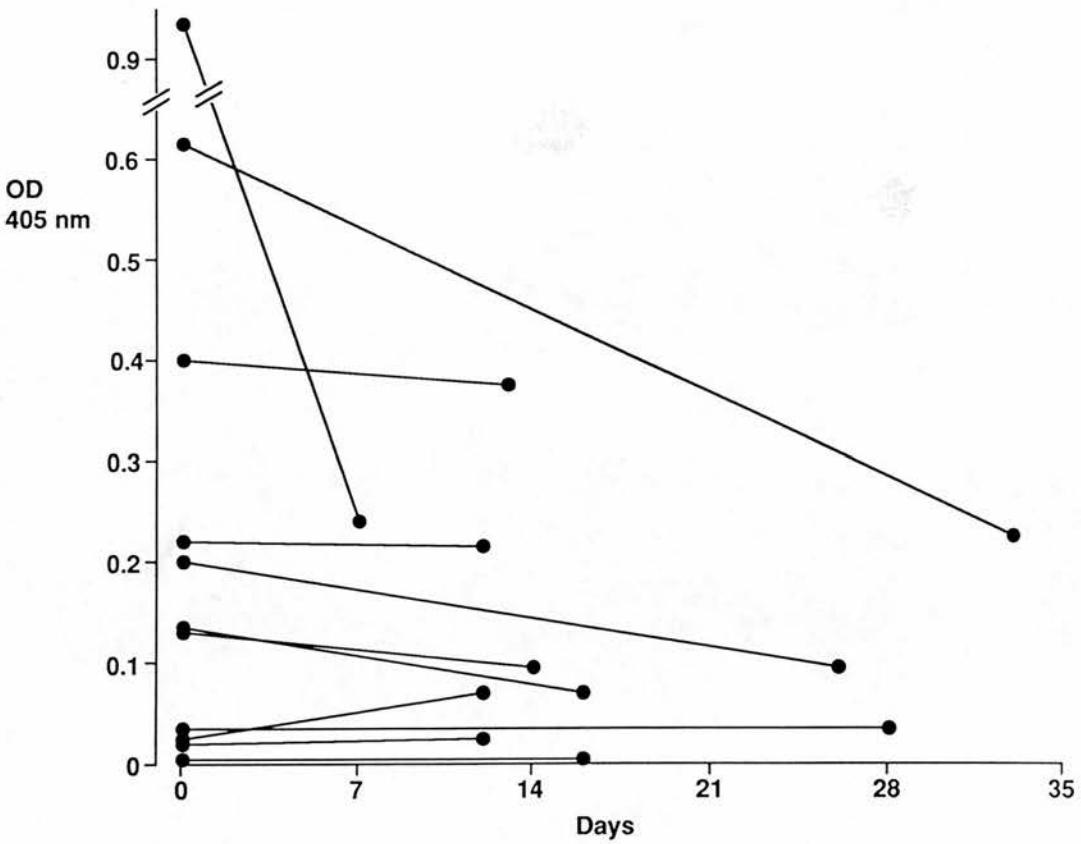
IgA serum antibody levels (OD readings) to egg and milk derived proteins, comparing healthy subjects who include with those who omit egg or milk from their diets. No significant differences.

Figure 5.10 **Dietary egg and milk, and specific serum IgM and IgG antibody levels.**



IgM and IgG serum antibody levels (OD readings) to egg and milk derived protiens, comparing healthy subjects who include with those who omit egg or milk from their diets. No significant differences (Mann-Whitney test).

Figure 5.11 **IgA anti-gliadin antibody levels in pure parotid saliva, and elemental diet.**



Change in IgA anti-gliadin levels (OD readings) in pure parotid saliva in subjects beginning an elemental diet for 7-34 days; n=11, no significant differences (Mann-Whitney test).

Table 5.1 **Correlation of protein, salivary flow, IgA, IgG, and IgM, in parotid saliva.**

	protein	IgA	IgG	IgM
flow	-0.119	-0.201	-0.129	-0.2
protein	-	0.088	0.192	0.276*

Rank correlation coefficients for various parameters within a stimulated five minute sample of pure parotid saliva in 92 subjects. Key: * p=0.008 (Spearman's coefficient).

Table 5.2 **Dietary egg, bread, and milk, and specific antibody levels in parotid saliva.**

	antibody isotype					
	IgA		IgG		IgM	
Food in diet	**Y**	**N**	**Y**	**N**	**Y**	**N**
Antibody to:						
Ovalbumin	16.5 (21)	8.2 (13)	0.65 (18)	1.75 (12)	0 (18)	0.34 (12)
	[0.21]		[0.09]		[1.0]	
ßlactoglobulin	8.7 (19)	5.0 (11)	0.1 (19)	2.6 (10)	0 (17)	0 (10)
	[0.13]		[0.077]		[0.42]	
Gliadin	3.15 (18)	3.0 (15)	2.5 (18)	6.0 (15)	0 (18)	0 (15)
	[0.89]		[0.19]		[1.0]	

Dietary antigen levels subdivided according to the inclusion of the major parent food in the diet, Y, or not, N (see chapter on methods). Median OD reading given, number of subjects in round brackets (n), and p value in square brackets, [x] (Mann-Whitney test).

CHAPTER SIX: SALIVARY IMMUNITY IN COELIAC DISEASE AND DERMATITIS HERPETIFORMIS

Introduction

Patients with coeliac disease or dermatitis herpetiformis are known to have increased immunity to food protein antigens in the systemic compartment of the immune system. There have in the past been some studies using insensitive techniques on antibody levels in intestinal fluids (mainly on jejunal juice - see chapter two), and a few more recent studies using sensitive ELISA methodologies (LaBrooy et al 1986; Volta et al 1988). These patients are an important target group, likely to have up-regulated secretory immunity to these food protein antigens which may be reflected in increased antibody levels in pure parotid saliva. If this was convincingly the case, there would be a potential use for parotid saliva as a screening, diagnostic, or monitoring tool.

Approach and results

Samples of pure parotid saliva and serum were obtained from control subjects and from patients with untreated and treated coeliac disease. All patients had recently undergone jejunal biopsy, are described in detail in chapter 3, and are summarised in table 6.1. Both pure parotid saliva and serum were assayed for total immunoglobulin concentrations and for specific antibodies of all three classes studied to gliadin, ovalbumin, and

ßlactoglobulin.

Table 6.1 **Subject details**

Subject	**M**	**F**	**total**	**age median (range)**		**smokers**
controls	13	15	28	25.5	(14-34)	6
coeliac disease						
- untreated	14	18	32	41.5	(16-78)	9
- treated	14	20	34	42.0	(15-72)	7
dermatitis herpetiformis						
- ND, NH	8	4	12	58.0	(27-86)	2
- ND, P/SVA	8	3	11	64.0	(15-83)	2
- GFD, NH	11	8	19	49.0	(28-82)	7
- GFD, P/SVA	15	3	18	52.5	(26-77)	5

Age (years), sex, and smoking habits of subjects. Key: ND = normal diet, GFD = gluten-free diet, NH = normal jejunal histology, P/SVA = partial or subtotal villous atrophy.

An opportunity also arose to study a group of well-characterised patients with dermatitis herpetiformis. These subjects had been investigated for other purposes, and had had a recent jejunal biopsy taken, and a dietary history for gluten obtained, along with a nutritional assessment, and some functional studies of small bowel integrity. Some had had serum samples taken one or two years prior to the present study, allowing comparisons over long period of time to be made. Again, full details of the group of subjects are included in chapter 3 and

summarised in table 6.1. Samples of pure parotid saliva and serum were obtained from these patients, and assayed for total immunoglobulin concentrations and for specific antibodies of all three classes studied to gliadin, ovalbumin, and ßlactoglobulin. The serum specimens were assayed for specific antibodies to gliadin and ovalbumin, and ßlactoglobulin of the IgA and IgG classes; serum total immunoglobulin concentrations have already been reported (Gawkrodger et al, 1988).

Coeliac disease

Total immunoglobulins.

There were no differences in total immunoglobulin concentrations in either saliva or serum when comparing control subjects to patients with untreated or treated coeliac disease (data not shown).

Specific antibodies

-saliva.

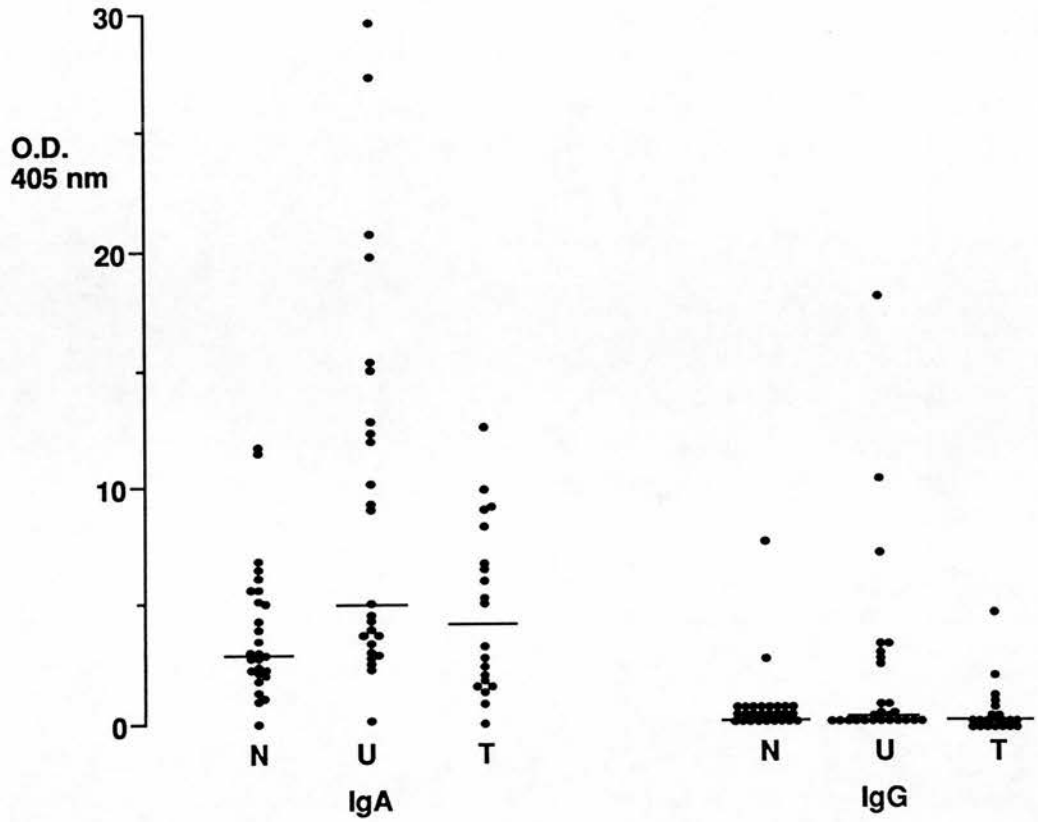
IgA anti-gliadin antibody levels were significantly elevated in the parotid saliva of patients with untreated coeliac disease when compared with patients with treated coeliac disease and control subjects, (table 6.2 and figure 6.1), with values in treated coeliac disease generally falling between those in untreated coeliac disease and controls. There were no significant differences in specific antibody levels to ovalbumin or ßlactoglobulin of any isotype (table 6.2).

Table 6.2 **Salivary antibody levels in coeliac disease**

Antibody	controls	untreated coeliac disease	treated coeliac disease
IgA-anti			
-gli*	3.0 (0-11.7)	5.1 (0.1-29.6)	4.15 (0-12.5)
-ova	14.1 (3-60.3)	15.8 (1.9-92.9)	13.2 (0.6-34)
-ßLG	6.4 (0-27.8)	8.3 (1.7-53.7)	8.3 (0-46.5)
IgG-anti			
-gli#	0 (0-4.6)	0.25 (0-17.9)	0 (0-7.6)
-ova**	0 (0-1.7)	0 (0-13)	0 (0-3.1)
-ßLG	0 (0-2.9)	4 (0-5.8)	0 (0-18.7)
IgM-anti			
-gli	2.4 (0-15.5)	5.05 (0-150)	5.42 (0-35.9)
-ova	1.15 (0-13.3)	1.22 (0-34.1)	1.35 (0-16.7)
-ßLG	1.2 (0-18.8)	1 (0-28.3)	1.2 (0-14.4)

Salivary antibody levels to gliadin (gli), ovalbumin (ova), and ß-lactoglobulin (ßLG) in control subjects, patients with untreated and treated coeliac disease. Values are median (range) OD readings. Key: p = * = 0.013; # = 0.005; ** = 0.025, (Kruskal-Wallis test).

Figure 6.1 **Salivary antibody levels in coeliac disease**



Antibody levels (OD readings; bar = median) to gliadin in pure parotid saliva in coeliac disease. Differences between groups are significant (IgA - p=0,013; IgG - p=0.005; Kruskal-Wallis test). Key: N = control, U = untreated coeliac disease, T = treated coeliac disease.

Specific antibodies

- serum.

Serum antibodies were exactly as described in the literature, with high anti-gliadin IgA and IgG antibodies found in patients with untreated coeliac disease, with low values in control subjects, and intermediate values found in patients with treated coeliac disease (table 3). There was a similar pattern with respect to IgA antibodies to ovalbumin and to ßlactoglobulin, and IgG antibodies to ßlactoglobulin (table 3).

Table 6.3 **Serum antibody levels in coeliac disease**

	controls	untreated coeliac disease	treated coeliac disease
Antibody			
IgA-anti			
-gli#	5.3 (0-44.5)	43.7 (4-150)	5.7 (0.3-42.9)
-ova*	10.95 (0.9-26)	18.83 (3-150)	14.75 (5-61)
-ßLG#	3.7 (0.15-36)	14.7 (1.4-150)	6.6 (2-87)
IgG-anti			
-gli#	20.95 (0-105)	82.43 (10-137)	41.58 (7-107)
-ova	36.05 (4-150)	64.58 (3-150)	63.4 (8-150)
-ßLG*	29.8 (1-136)	94.7 (5-150)	70.2 (9-150)
IgM-anti			
-gli	47.85 (16-109)	43 (19-95)	38.6 (10-88)
-ova	30.1 (8-103)	31.1 (4-87)	37.8 (9-123)
-ßLG	28.1 (10-82)	33.57 (9-125)	38.72 (11-95)

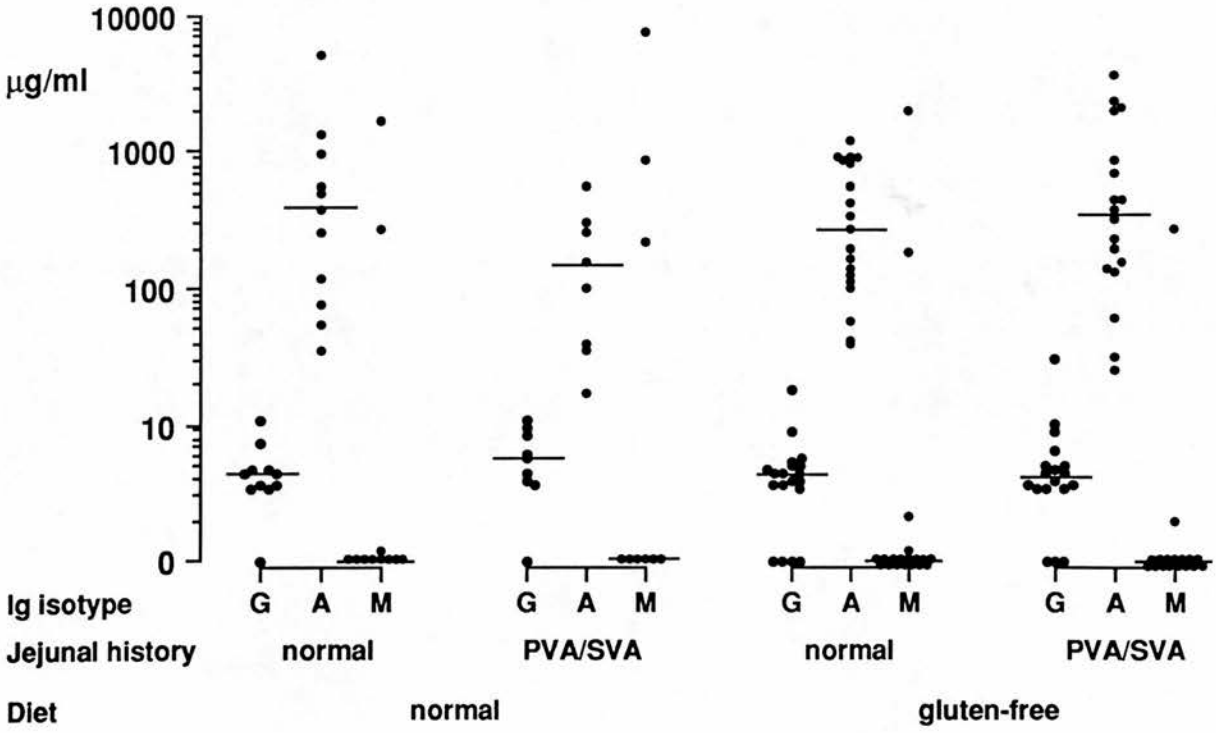
Serum antibody levels to gliadin (gli), ovalbumin (ova), and ß-lactoglobulin (ßLG) in control subjects, patients with untreated and treated coeliac disease. Values are median (range) OD readings. Key: p = * <0.01, # <0.001, (Kruskal-Wallis test).

Dermatitis herpetiformis

Total immunoglobulins.

There were no differences in total parotid salivary immunoglobulin concentrations between the groups of patients with dermatitis herpetiformis who differed in respect of jejunal histology and dietary gluten intake (figure 6.2).

Figure 6.2 **Salivary immunoglobulin concentrations in dermatitis herpetiformis**



Total immunoglobulin concentrations (bar = median) in parotid saliva of patients with dermatitis herpetiformis, with either normal jejunal histology or partial (PVA) or subtotal villous atrophy (SVA), stratified according to normal or gluten-free diet. No significant differences between groups, (Kruskal-Wallis test).

Specific antibodies

- saliva.

In general there were no significant differences in specific salivary antibody levels, of any of the three isotypes studied when comparing patients with and without enteropathy irrespective of their being on a normal or on a gluten-free diet (table 6.4). There were frequently strong positive correlations between the levels of antibody to different antigens in parotid saliva, and between different isotypes of antibody to a single antigen, (table 6.5).

- serum.

With respect to serum there were no differences between antibody levels to any of the three antigens studied when comparing all groups of patients (table 6.6). There were frequently strong positive correlations between antibody levels to one antigen and to another antigen, irrespective of antibody isotype, (table 6.7), just as found in saliva. In paired serum samples taken one to two years apart, when there was no change in diet, antibody levels remained remarkably constant (for example IgA anti-gliadin antibodies, figure 6.3). When diet changed from a normal, gluten-containing, one to a gluten-free diet, IgG anti- gliadin antibody levels were significantly lower in the second sample than one year previously, $p=0.045$, (figure 6.4), however all other antibody levels remained constant, (data not shown).

Table 6.4 **Salivary antibody levels in dermatitis herpetiformis**

Diet	**normal**		**gluten-free**	
Jejunal histology	**N**	**P/SVA**	**N**	**P/SVA**
Antibody				
IgA-anti				
-gli	0.39 (0-0.59)	0.24 (0-0.5)	0.29 (0-1.01)	0.31 (0.1-1.5)
-ova	0.22 (0-1.5)	0.2 (0-1.25)	0.42 (0.1-1.2)	0.53 (0.1-1.5)
-ßLG	0.22 (0-1.5)	0.1 (0-0.41)	0.15 (0-1.15)	0.5 (0.1-1.5)
IgG-anti				
-gli	0 (0-0.8)	0 (0-0.13)	0 (0)	0 (0-0.15)
-ova	0 (0-1.5)	0 (0-0.16)	0.28 (0-1.4)	0.23 (0-1.2)
-ßLG	0 (0)	0 (0-0.13)	0 (0-0.1)	0 (0-0.1)
IgM-anti				
-gli	0 (0-0.28)	0 (0-0.2)	0.016 (0-0.26)	0.142 (0-1.05)
-ova	0.02 (0-0.25)	0 (0-0.19)	0.07 (0-0.55)	0.08 (0-1.5)
-ßLG	0 (0-0.1)	0 (0-0.14)	0 (0-0.31)	0 (0-1.4)

Median OD (range) of pure parotid salivary antibody levels in patients with dermatitis herpetiformis, according to diet and histology. No significant differences between groups, (Kruskal-Wallis test). Key: N = normal, P/SVA = partial or subtotal villous atrophy.

Table 6.5 **Correlations of salivary antibody levels in dermatitis herpetiformis**

Clinical group			gliadin		ova		ßLG	
			IgA	IgG	IgA	IgG	IgA	IgG
ND, NH	gliadin	IgA						
		IgG						
	ova	IgA						
		IgG						
	ßLG	IgA						
		IgG						
GFD, NH	gliadin	IgA						
		IgG						
	ova	IgA						
		IgG	.71#	.59*	.59*			
	ßLG	IgA			.57*			
		IgG	.58*					
ND, S/PVA	gliadin	IgA						
		IgG						
	ova	IgA	.86#					
		IgG						
	ßLG	IgA	.92##		.98##			
		IgG				.98##		
GFD, S/PVA	gliadin	IgA						
		IgG						
	ova	IgA	.76#					
		IgG						
	ßLG	IgA	.61*		.71#			
		IgG				.65**		

Spearman rank correlation coefficients between antibody levels in saliva in patients with dermatitis herpetiformis, stratified according to diet and jejunal histology. Only statistically significant correlations between antibody levels within saliva are shown for clarity. Key: ND = normal diet, GFD = gluten-free diet, NH = normal jejunal histology, P/SVA = partial or subtotal villous atrophy, * = p = <0.05, ** = <0.02, # = <0.01, ## = <0.001.

Table 6.6 **Serum antibody levels in dermatitis herpetiformis**

Diet	**normal**		**gluten-free**	
Jejunal histology	**N**	**P/SVA**	**N**	**P/SVA**
Antibody				
IgA-anti				
-gli	0.035 (0-0.29)	0 (0-1.6)	0.04 (0-0.79)	0.08 (0-0.59)
-ova	0.17 (0-1.26)	0 (0-0.38)	0.15 (0-0.48)	0.17 (0-1.3)
-ßLG	0 (0)	0 (0)	0 (0)	0 (0)
IgG-anti				
-gli	0.18 (0-4.7)	0 (0-1.26)	0.1 (0-0.68)	0.1 (0-1.5)
-ova	0.03 (0-1.02)	0 (0-1.3)	0.057 (0-1.5)	0.05 (0-0.97)
-ßLG	0 (0)	0 (0)	0 (0)	0 (0)

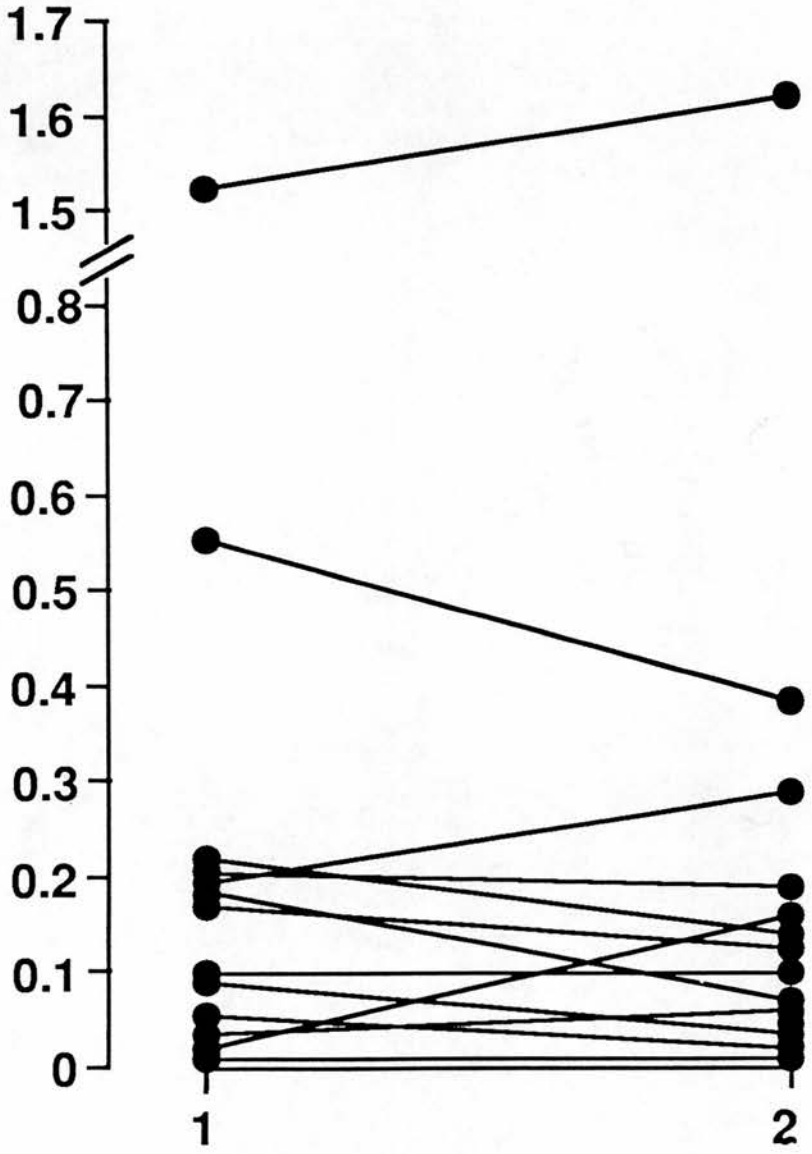
Median (range) of serum antibodies in OD readings in patients with dermatitis herpetiformis stratified according to diet and jejunal histology. No significant differences between groups, (Kruskal-Wallis test). Key: ND = normal diet, GFD = gluten-free diet, NH = normal jejunal histology, P/SVA = partial or subtotal villous atrophy.

Table 6.7 **Correlations of serum antibody levels in dermatitis herpetiformis**

Clinical group			gliadin		ova	
			IgA	IgG	IgA	IgG
ND, NH	gliadin	IgA				
		IgG	.805#			
	ova	IgA	.818#	.907##		
		IgG		.761#		
GFD, NH	gliadin	IgA				
		IgG	.74#			
	ova	IgA	.85##	.68#		
		IgG	.69#	.72#	.76#	
ND, S/PVA	gliadin	IgA				
		IgG	.99##			
	ova	IgA	.94##	.93##		
		IgG	.93##	.92##	.95##	
GFD, S/PVA	gliadin	IgA				
		IgG	.8#			
	ova	IgA	.93##	.85##		
		IgG	.83##	.75*	.86##	

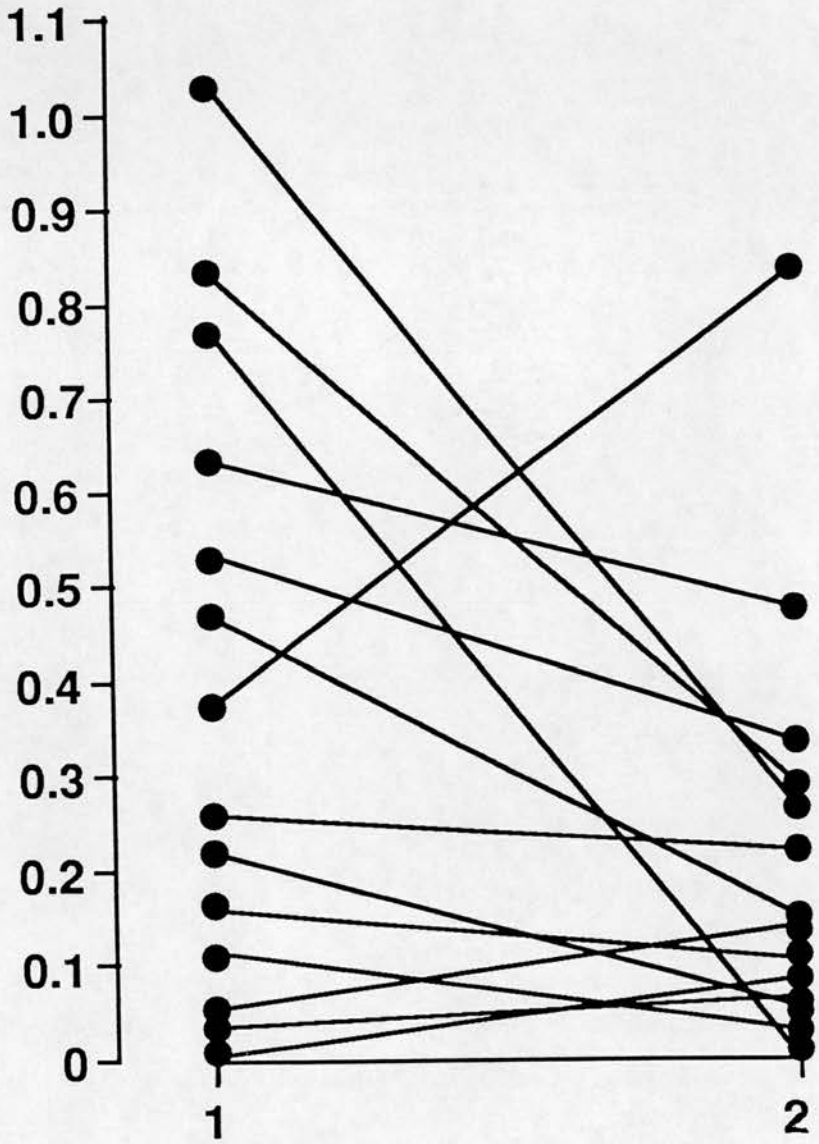
Spearman rank correlation coefficients between antibody levels in serum in patients with dermatitis herpetiformis, stratified according to diet. Only statistically significant correlations between antibody levels within serum are shown for clarity. Key: ND = normal diet, GFD = gluten-free diet, NH = normal jejunal histology, P/SVA = partial or subtotal villous atrophy, * = p = <0.05, ** = <0.02, # = <0.01, ## = <0.001; ova = ovalbumin.

Figure 6.3 **Change in anti-gliadin antibody levels with no change in diet**



Serial serum IgA anti-gliadin antibody levels (OD readings, 405nm) taken one year apart in patients with dermatitis herpetiformis with a normal jejunal mucosa, on a normal diet. N = 14, no significant change in levels, (Mann-Whitney test).

Figure 6.4 **Change in serum antigliadin antibodies with change in diet.**



Fall in serial serum IgG anti-gliadin antibody levels (OD readings, 405nm) taken one year apart in patients with dermatitis herpetiformis with villous atrophy, after changing to a gluten-free diet. N = 14, p = 0.018, (Mann-Whitney test).

Conclusions and implications

As predicted salivary antibody levels were high in untreated coeliac disease and fell in treated coeliac disease, parallelling changes in serum antibody levels. There was a lot of overlap in the levels however, and this probably precludes the use of saliva as a diagnostic tool in a clinical setting, or for monitoring therapy. Surprisingly there was no detectable influence of jejunal histology or dietary gluten intake on salivary antibody levels in patients with dermatitis herpetiformis. Antibody levels to gliadin (and to other antigens) were maintained despite avoiding gluten on a gluten-free diet, and despite mucosal healing which suggests good dietary compliance, and that the persistent antibody levels were not due to continued intake of (smaller amounts) of antigen. These findings suggest that there is mucosal B-cell memory for dietary antigens.

As expected total immunoglobulin concentrations in both serum and saliva in control subjects, in patients with coeliac disease, and in patients with dermatitis herpetiformis, were similar. The frequency of strongly positive correlations between levels of different antibodies in saliva suggests non-specific activation of the humoral immune response. This would suggest that the high antibody levels seen are a secondary response rather than being pathologically involved in the primary disease process. This may also hold true for serum antibody levels. The lack of any correlation in the absence of a

co- existing or previous enteropathy suggests that a breach of the mucosa is necessary at some stage to initiate a systemic antibody response. The persistence of virtually all serum antibodies, either on the same diet or even after change to a gluten-free diet further supports the hypothesis that there is B cell memory for these protein antigens.

CHAPTER SEVEN: IMMUNOGLOBULIN AND ANTIBODY LEVELS IN LAVAGE FLUID

INTRODUCTION AND AIMS

Having established reliable methods for collection and assay of intestinal fluid by gut lavage, healthy individuals were used to answer specific questions. The opportunity also arose to study a large number of patients with coeliac disease. Since these patients are known to have increased systemic immunity to food protein antigens, it might be expected that they would also have increased secretory immunity. Indeed some early studies using insensitive methods (Douglas et al, 1970; Lancaster-Smith et al, 1974), and one or two more recent investigations using ELISA on jejunal fluid (LaBrooy et al, 1986; Volta et al, 1988) support this theory. As many such patients already attended the gastrointestinal unit for investigation and treatment, some were invited to undergo lavage and give saliva.

The specific questions posed were, firstly, what were the concentrations of total immunoglobulins in lavage fluid?; secondly, what proportion of IgA in lavage in health is dimeric?; thirdly, could antibodies to food protein antigens be detected in lavage fluid?; fourthly, did the profound effects of smoking on parotid salivary immunity extend to intestinal lavage fluid?; and finally, were there abnormalities in immunoglobulin concentrations and specific antibody levels in coeliac disease?

APPROACH

Healthy non-smoking and smoking volunteers were approached and asked to undergo intestinal lavage. Patients with untreated and treated coeliac disease attending the gastrointestinal unit for investigation were also invited to have intestinal lavage. In addition the opportunity to study a group of patients with inactive ulcerative colitis also arose. These patients had already had gut lavage as part of a separate study, on bacterial and candidal antibodies, undertaken by ourselves in collaboration with Dr. E.D. Srivastava (University Hospital of Wales, Cardiff). Samples of intestinal fluid from these patients were therefore available for assay.

The details of the administration, collection, and treatment of the lavage fluid are given in chapter four. All samples were assayed for total IgA, IgM, and IgG, and for isotype-specific antibodies to gliadin, ovalbumin, and ßlactoglobulin. In addition, samples from healthy volunteers were assayed for dimeric (secretory) IgA. This assay was set up towards the end of the thesis, and was therefore only applied in a small number of studies.

RESULTS

Total immunoglobulins.

The normal range of total immunoglobulins is shown in table 7.1. IgA was found in the highest concentration, with small but detectable concentrations of IgM and IgG present. In patients with untreated and treated coeliac disease there were no differences in IgA or IgG concentration, but IgM was significantly increased in both patient groups when compared with healthy volunteers, (table 7.1).

The proportion of IgA in healthy volunteers which was dimeric was a median of 92% (range 82.3-100).

Table 7.1 **Lavage fluid immunoglobulin concentrations**

	IgA	IgM	IgG
Controls (n=25)	139.7 (7.9-403)	5.3 (0-37)	1.0 (0-12.2)
Coeliacs			
untreated (n=15)	146.2 (10.7-487)	17.5* (2.1-100)	2.7 (0.1-34.9)
treated (n=19)	90.8 (8.8-621)	25.7* (0-192.2)	0.9 (0.1-11.4)

Median (range) of total immunoglobulin concentrations in ug/ml in intestinal lavage fluid. Key: * = significantly different from control values, $p<0.05$, (Mann-Whitney test).

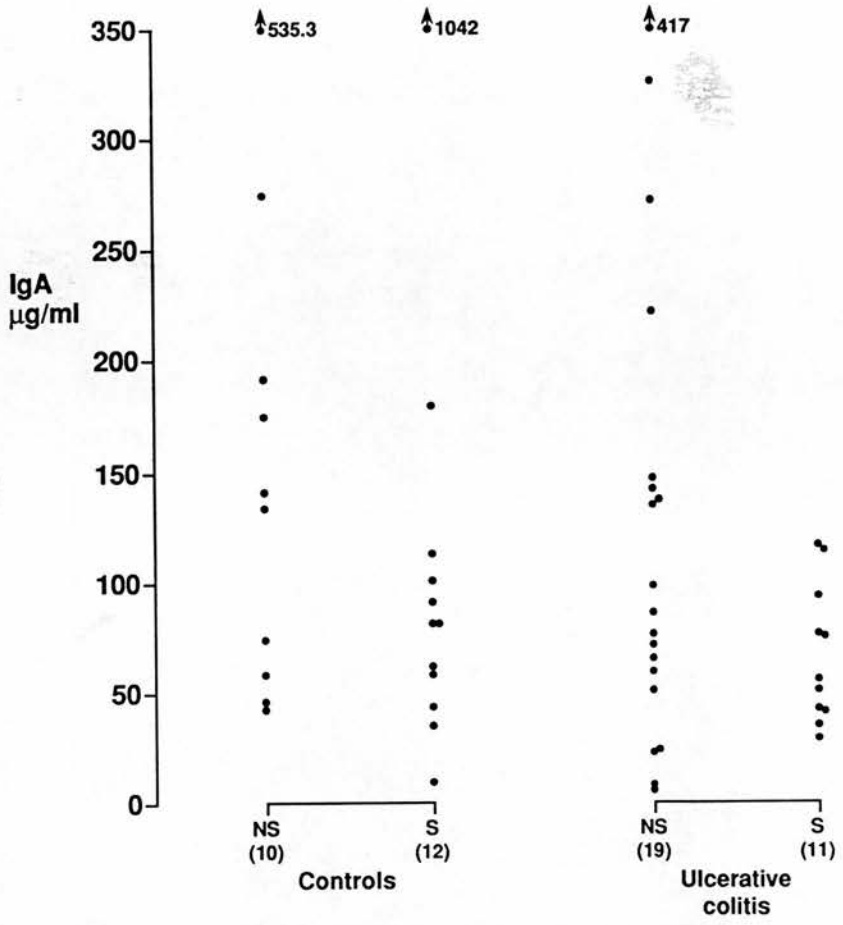
Specific antibodies.

Antibodies to gliadin, ovalbumin, and ßlactoglobulin, of all three isotypes tested, were regularly detected in healthy subjects, and were usually at low levels. IgA and IgM specific antibodies to all three food protein antigens were increased in patients with coeliac disease; antibody levels in treated patients were often intermediate between those in healthy volunteers and those in patients with untreated coeliac disease. There were no differences in IgG antibodies (table 7.2).

Smoking.

In both healthy volunteers and in patients with inactive ulcerative colitis, smoking decreased total IgA concentrations, but these changes did not reach statistical significance, (figure 7.1). There were no differences in total IgM or IgG concentrations, nor in specific antibody levels, when comparing non-smokers with smokers. Only three subjects smoked more than twenty cigarettes daily (two controls, one patient).

Figure 7.1 **Lavage IgA concentrations and smoking**



Total IgA concentrations (median) in lavage fluid in healthy controls and in patients with inactive ulcerative colitis, subdivided by smoking habits. No significant differences between groups, (Kruskal-Wallis test). Key: NS = non-smokers, S = smokers.

CONCLUSIONS AND IMPLICATIONS

A normal range for total immunoglobulin concentrations and specific antibody levels in gut lavage fluid has been established. Total IgA is largely dimeric in the lavage fluid of healthy individuals. Total IgM concentrations, and specific IgA and IgM antibodies are increased in coeliac disease, not only to gliadin but also to ovalbumin and ßlactoglobulin. Smoking may decrease IgA in lavage fluid; further studies, on heavy smokers, are required.

Table 7.2 **Lavage fluid antibody levels**

	Gliadin	**ßlactoglobulin**	**ovalbumin**
IgA			
Controls	2.2 (0-58)	3.5 (0.1-71)	4.8 (0-76)
Coeliac			
- untreated	53.6# (2.2-138)	7.9* (1.8-85)	15* (3.7-39)
- treated	9.2* (0.4-73)	8.4 (0.6-76)	14.7* (0.5-47)
IgM			
Controls	3.1 (0-89)	0.1 (0-23)	0.9 (0-21)
Coeliac			
- untreated	56.9# (8-150)	5.6* (0-14)	6.6* (0.8-33)
- treated	17.9* (0.2-150)	5.3* (0-53)	3.6* (0-46)
IgG			
Controls	0.2 (0-6)	0.4 (0-4)	0 (0-4)
Coeliac			
- untreated	0.4* (0.2-19)	0.7 (0-21)	0.3 (0-1)
- treated	0.6 (0-3)	0.4 (0-8)	0 (0-61)

Median (range) of antibody levels (OD readings) in intestinal lavage fluid. Key: # = significantly different from both treated patients and controls, * = significantly different from control subjects, $p<0.05$ for both comparisons, (Mann-Whitney test).

CHAPTER EIGHT: RELATIONSHIPS OF ANTIBODIES AND OF IMMUNOGLOBULINS IN SECRETIONS AND IN SERUM.

INTRODUCTION

The mucosal and systemic immune systems are distinct and separate (Chapter 2). Within the overall immune response of an individual possible direct or inverse relationships between secretions, either for isotype pattern, or specific antibodies, have been little examined. Animal models suggest that after feeding of antigen there is induction of mucosal antibodies, and active down-regulation of systemic immunity (Challacombe, 1983). Thus, generally there seems to be an inverse correlation. In contrast, after parenteral injection of antigen, serum antibody formation usually follows without secretory antibodies (Baumann et al 1985; Smith et al 1986). This is probably due to a lack of any effect rather than active suppression of the secretory antibody response. Although there is much evidence that all areas of the mucosal immune system are linked (thus, the appellation "common mucosal immune system"), the relationships between antibody levels in various secretions are uncertain. The small number of studies so far have given conflicting results (see Chapter 2). The aims of this section were

1. By comparing salivary with serum total immunoglobulin concentrations, and antibody levels, to establish whether there is a direct or inverse relationship between

the gut associated lymphoid tissue and the systemic immune system, or, possibly, no relationship at all.

2. To extend this question to the relationship between whole gut lavage and serum, and jejunal aspirate and serum.

3. To assess the value of saliva as a mirror of immune events in more distal gut secretions by comparing saliva to whole gut lavage, and saliva to jejunal aspirate, and, for completeness, comparing jejunal aspirate to whole gut lavage.

APPROACH

Using data from four groups of subjects described in Chapter 3 and for whom details are shown in Table 1, non-parametric correlation coefficients were determined for immunoglobulin concentrations and specific antibody titres in the four fluids under study within each group. As this necessitated multiple statistical analyses on the correlations only those correlations with a probability of less than 1% ($p<0.01$) were taken to be significant.

Table 8.1 **Subject details**

Subject		M (n)	F (n)	total (n)	age (yrs) median	(range)
Controls						
ser/sal vs	- lav	9	16	25	53	(21-92)
	- asp	13	15	28	25.5*	(14-84)
Coeliac disease						
Untreated						
ser vs	- sal	14	18	32	41.5	(16-78)
ser/sal vs	- asp	12	14	26	39.5	(16-78)
	- lav	6	9	15	43	(28-62)
Treated						
ser vs	- sal	14	20	34	42	(15-72)
ser/sal vs	- asp	9	13	22	42	(15-72)
	- lav	8	11	19	46	(16-72)
Dermatitis herpetiformis						
NH, GFD		10	11	21	52	(27-82)
P/SVA, ND		12	7	19	54	(15-83)
P/SVA, GFD		17	4	21	52	(26-80)
Heterogeneous subjects						
		10	14	24	45	(22-72)

Demographic details of subjects. Key - ser = serum, sal = saliva, lav = lavage, asp = jejunal aspirate, ND = normal diet, GFD = gluten-free diet, NH = normal jejunal histology, S/PVA = subtotal or partial villus atrophy. Controls having jejunal aspirate younger than other groups, * $p<0.05$, (Mann-Whitney test), no other significant differences.

RESULTS

The main results are shown diagrammatically in Table 8.2. Overall there were few highly significant correlations. With regards to the comparison of serum and parotid saliva significant correlations were present and the relationships were usually direct and positive. Such correlations were seen most frequently when there was, or had been, an enteropathy present. Additionally, in these patients there were positive correlations between antibody levels in serum and antibody levels in both lavage and jejunal fluids.

Serum IgG antibodies (within a clinically cohesive group of patients) correlated positively with salivary IgG antibodies and also with salivary IgA antibodies to the same antigen. Positive correlations were most noticeable in patients with untreated coeliac disease with villus atrophy, and in patients with treated dermatitis herpetiformis with a normal jejunal mucosa. Conversely, no such correlations were demonstrable in patients with treated coeliac disease and patients with dermatitis herpetiformis with villus atrophy (Table 8.3).

Generally, within an individual, pure parotid salivary immunoglobulin concentrations and specific antibody levels did not reflect those in jejunal aspirate or whole gut lavage fluid. Although a few significant correlations emerged there was no overall pattern apparent. There

Table 8.2 **Correlations of immunoglobulins and of antibodies between serum, saliva, aspirate, and lavage fluid**

ser	sal	asp	lav	IgA tot	IgA gli	IgA ßlg	IgA ova	IgM tot	IgM gli	IgM ßlg	IgM ova	IgG tot	IgG gli	IgG ßlg	IgG ova
controls															
*	*			.46		-.41		.48				.37	.54		
*		*							.64*						
*			*												
	*	*													
	*		*												
coeliacs															
untreated															
*	*							.45					.42		
*		*			.65*	.56*								.56*	.51
*			*		.53	.60							.77*		
	*	*				.48		.58*							
	*		*												
treated															
*	*							.63*					.63*		
*		*										-.45			
*			*												
	*	*							.69*						
	*		*												
dermatitis herpetiformis															
NH, ND															
*	*						.69								
NH, GFD															
*	*						.7*								.68*
S/PVA, ND															
*	*												.62		
S/PVA, GFD															
*	*														.56

Rank correlation coefficients (with p values <0.05) between total immunoglobulin concentrations, and between specific antibodies in serum (ser), saliva (sal), jejunal aspirate (asp), and lavage (lav). Comparisons being made as shown by asterisks joined by solid lines in left-hand column. Aspirate and lavage were only available for comparison in the heterogeneous group (table 4). Key: tot = total immunoglobulin, gli= gliadin, ova = ovalbumin, ßlg = ßlactoglobulin, NH = normal jejunal histology, S/PVA = subtotal or partial villous atrophy, ND = normal diet, GFD = gluten-free diet, * = $p<0.01$.

were a couple of weakly positive correlations between immunoglobulins or antibody levels which failed to reach the statistical criterion of $p<0.01$, when comparing jejunal aspirate and lavage fluid, although this heterogeneous group mainly consisted of patients with untreated coeliac disease (Table 8.4).

Table 8.3 **Correlation between serum IgG and salivary IgA**

Subjects	**gli**	**ova**	**ßlg**
controls			
coeliac disease			
untreated	0.615		
treated			
dermatitis herpetiformis			
NH, GFD	0.609*	0.603*	
S/PVA, ND			
S/PVA, GFD			

Spearman rank correlation coefficients (with p values <0.05) between serum IgG and salivary IgA specific antibodies to gliadin (gli), ovalbumin (ova), and ßlactoglobulin (ßlg). Key: * = p <0.01, NH = normal jejunal histology, S/PVA = sub-total or partial villous atrophy, ND = normal diet, GFD = gluten-free diet.

Table 8.4 **Correlations between saliva, aspirate, and lavage**

secretion			IgA			IgM	IgG
sal	asp	lav	tot	gli	ßlg	ova	
*	——	*					no
*	————	*	.473				significant
	*	—— *	.463				correlations

Spearman rank correlation coefficients (with p values <0.05) for total immunoglobulins (tot), and specific antibodies to gliadin (gli), ovalbumin (ova), and ßlactoglobulin (ßlg) in saliva (sal), jejunal aspirate (asp), and gut lavage (lav) in heterogeneous group of subjects, (n=24). No correlations with p value <0.01 were found.

CONCLUSIONS

Most commonly there were no relationships between total immunoglobulin concentrations or between antibody levels when comparing parotid saliva and serum. On the infrequent occasion where a relationship was apparent, gut mucosal damage often was, or had been, present. Such a relationship was usually direct (positive). Similarly, the correlations between serum and either jejunal aspirate or lavage fluid were infrequent. Parotid saliva did not seem to reflect, in an individual at least, immune events in jejunal aspirate or in gastrointestinal lavage fluid.

CHAPTER NINE: THE EFFECTS OF SMOKING ON SALIVARY IMMUNITY

Introduction

Cigarette smoking is not only known to have profound effects on the immune system, but in addition there is strong epidemiological evidence that smoking has a role in certain inflammatory and malignant oro-gastro-intestinal diseases. To investigate a possible effect of smoking on mucosal immunity saliva which had been collected in Edinburgh by Dr. Magdy Riad for other purposes (see chapter 3) from healthy smokers, healthy non-smokers, and patients with epithelial head and neck tumours was obtained and analysed. Results from these Scottish patients and healthy subjects revealed significant differences between the various groups. To confirm this data and in addition to avoid the possible confounding effects of concurrent alcohol consumption, prospective studies were then performed in Egyptian, non-drinking, healthy subjects and patients with head and neck tumours. To assess whether or not the alterations in immunoglobulins were unique to head and neck tumours, tumours local to the salivary glands, a group of patients with tumours in other regions was studied.

In some patients in the initial Edinburgh study who had recently completed a course of radiotherapy very high levels of total IgM were detected. To fully assess the influence of radiotherapy on immunoglobulin and specific

antibody levels, a prospective study of patients undergoing a course of treatment in Edinburgh was undertaken. The studies on parotid salivary fluid demonstrated profound effects on immunoglobulin concentrations, and therefore parotid gland specimens were subsequently examined to assess plasma cell numbers.

SUBJECTS AND METHODS

Edinburgh healthy subjects and patients

Numbers of subjects and clinical details are given in chapter 3, and in tables 3.5 and 3.6 in that chapter. Due to the retrospective nature of this phase of the study alcohol intake was not recorded, smoking history was incomplete for the non-smokers (never smoked or ex-smokers?) and the groups were not age or sex matched. The patients in the prospective arm of the study undergoing radiotherapy had the same standard course of treatment as those in the retrospective arm (see chapter 3). Six of the 14 patients were current or ex-smokers, with 8 stating that they had never smoked.

Cairo healthy subjects and patients

Clinical details of these subjects are given in chapter 3, tables 3.2-3.4. Of the Cairo volunteers and patients the mean age of the smokers and non-smoking controls was not significantly different ($p=0.682$). Similarly, the tumour patients and the age- and sex-matched controls were not different in terms of age ($p=0.596$). The irradiated group were somewhat older, mean 59 years, than both

the control, 52 years, and the tumour patients, 53 years, (p=0.0004 vs controls; p=0.0033 vs tumour patients; all tests Mann-Whitney). These patients had tumours in various sites in the head and neck (table 3.7, chapter 3) which did not differ significantly between groups, (chi-squared =0.699, 2 df, p=0.71).

There were no differences in the sex distribution between any group of Egyptian subjects, (chi-squared = 3.164, 7df, p=NS).

Clinical procedures.

Collection of saliva was bilateral and simultaneous for two minutes (Cairo) or unilateral for five minutes (Edinburgh group), but otherwise followed the protocol outlined in chapter 4.

The assays of salivary total proteins and immunoglobulin concentration are described in appendices (i) and (v). Salivary electrolytes were analysed on a standard SMAC II.

PLASMA CELL NUMBERS IN PAROTID GLANDS.

Subjects and methods.

Parotid gland specimens were obtained from cadavers undergoing post-mortem at the Western General Hospital with the co-operation of Dr Margaret McIntyre, consultant pathologist. Initial immunoperoxidase staining of the first few specimens revealed marked background staining

which seemed likely to be due to autolytic artifact. Therefore, fresh, per-operative specimens were obtained from patients undergoing parotidectomy for parotid adenoma, in co- operation with Professor AGD Maran of the department of Otolaryngology. Only normal parotid tissue was examined. Each specimen was processed as described in appendix (iii). Counts of isotype-specific plasma cells were made under a magnification of x400, and were enumerated per ten high power fields. Each specimen was assessed in a blind manner, and each was counted twice, again blindly. A comparison of the plasma cell counts between smokers and non-smokers was made.

RESULTS

SALIVA.

1. Edinburgh - retrospective study.

The results are shown in table 9.1. There were no differences between the disease control groups of patients with inactive thyroid disease, anorexia, bulimia, and obesity, and the control group, and all these subjects were considered together as a large control population for statistical analysis. Salivary IgA concentrations were decreased in smokers, and in patients with head and neck tumours, both before and after irradiation. Conversely,

Table 9.1 **Salivary immunoglobulin concentrations**

	Subjects			
	non-smokers	**smokers**	**tumour patients**	**irradiated patients**
	(n=114)	(n=28)	(n=27)	(n=30)
IgA	145±92	44±41*	80±61*	87±49*
IgM	0.26±0.4	1.04±0.5*	0.08±0.2	3.2±9.2
IgG	3.7±9.5	0.07±0.1	I	I

Mean (±sd) salivary immunoglobulin concentrations in ug/ml in the Edinburgh subjects. Key: * = significant difference from control values, $p<0.0005$ in all cases, (Mann-Whitney test). I = insufficient saliva.

IgM concentrations were increased in smokers, and in patients with head and neck tumours, with a few extremely high values in the post-irradiation group. These results must be interpreted with caution as the age and sex distribution was distinctly different from the healthy subjects and disease controls, who were a heterogeneous group.

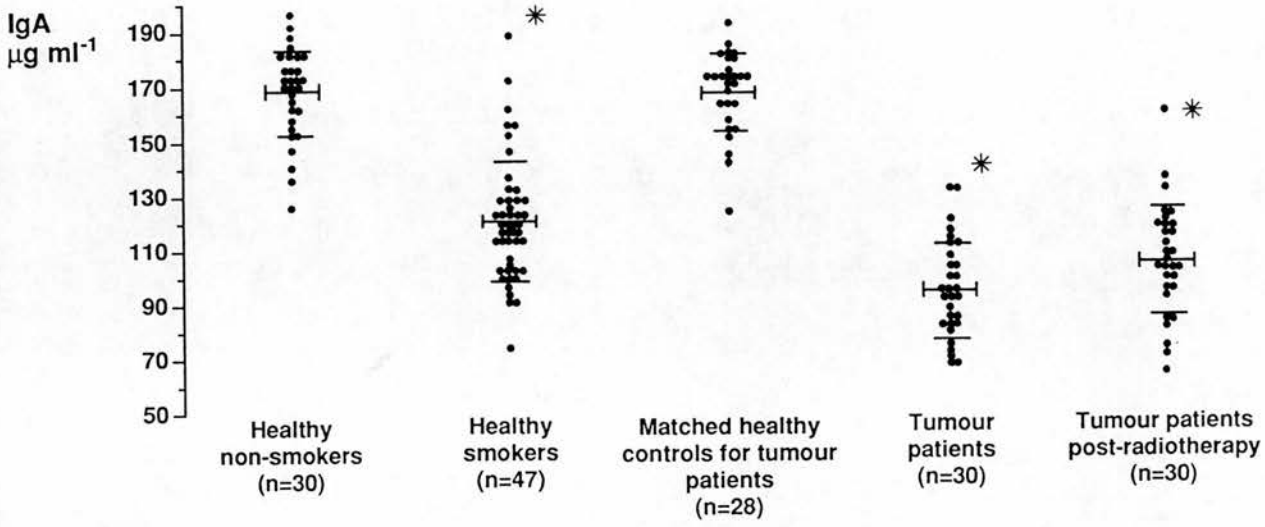
2. Edinburgh - prospective study: head and neck tumours.

There were no significant changes in total immunoglobulin or specific antibody levels with the course of radio-therapy (results not shown).

3. Cairo

Healthy smokers had significantly lower salivary IgA concentrations (Figure 9.1), and higher salivary IgM

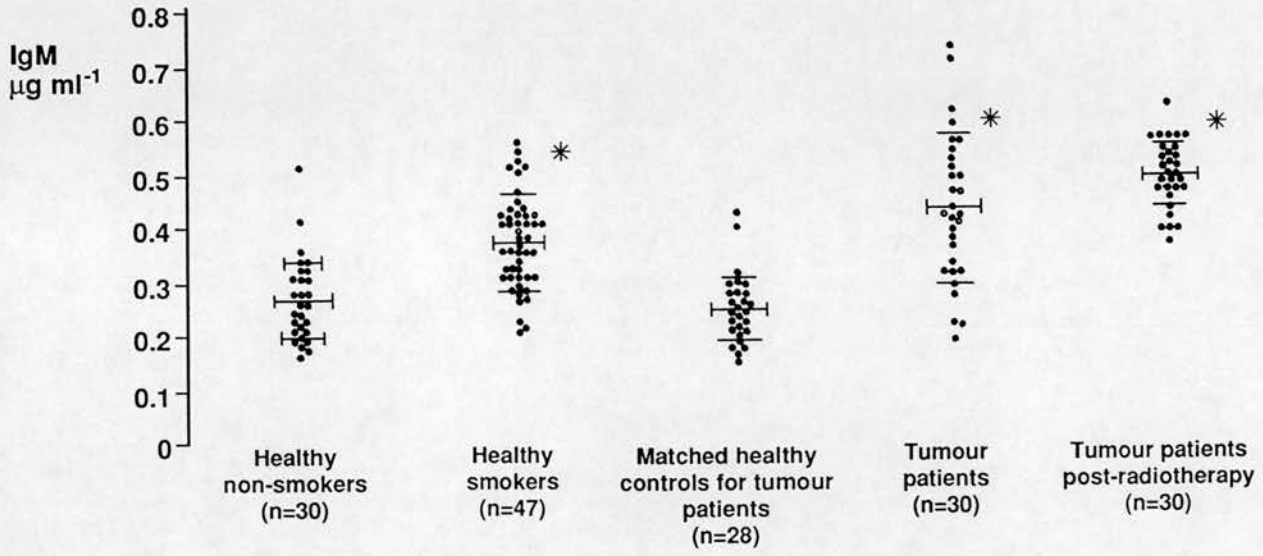
Figure 9.1 **Smoking and salivary IgA concentrations**



Parotid salivary IgA concentrations in Cairo subjects. Key: * = significant difference between appropriate control groups and smokers and patients ($p<0.00001$ vs healthy smokers; $p<0.00001$ vs tumour patients; $p<0.00001$ vs irradiated tumour patients, Mann-Whitney test). Bars show mean ± standard error.

(Figure 9.2), when compared with non-smokers. There was no influence of smoking on salivary IgG concentration, (figure 9.3). There was a strong inverse correlation between salivary IgA concentration and the number of cigarettes currently smoked daily (10-60), (Figure 9.4); however, no such relationship existed for salivary IgG, or IgM concentrations. Salivary flow rates were weakly but significantly correlated positively with the number of cigarettes smoked daily ($r=0.291$, $p=0.044$, Spearman correlation coefficient) but no relationship existed between salivary immunoglobulin concentrations and flow rate.

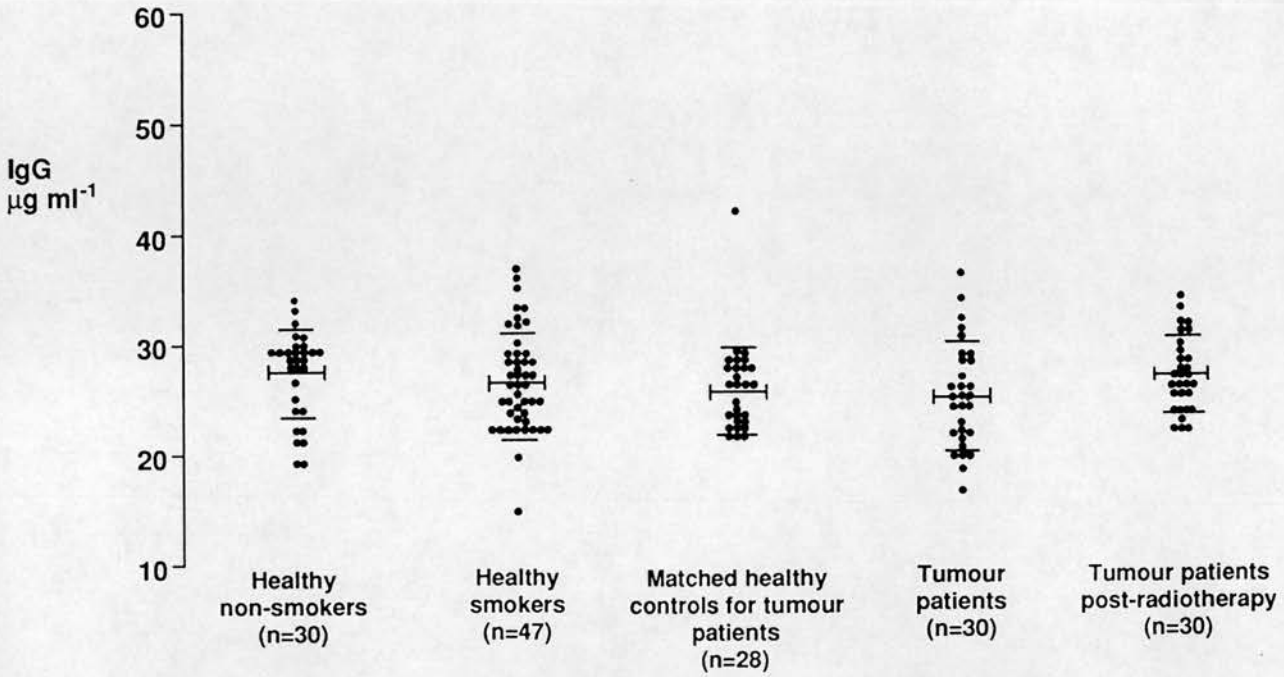
Figure 9.2 **Smoking and salivary IgM concentrations**



Parotid salivary IgM concentrations in Cairo subjects. Key: * = significant difference between appropriate control groups and smokers and patients ($p<0.00001$ vs healthy smokers; $p<0.00001$ vs tumour patients; $p<0.00001$ vs irradiated tumour patients; Mann-Whitney test). Bars show mean ± standard error.

Salivary IgA concentrations in ex-smokers of at least 20 cigarettes daily, who had given up smoking two years prior to sampling, were higher than in healthy current smokers, and after five years of non-smoking IgA concentrations had returned to those comparable with those of

Figure 9.3 **Smoking and salivary IgG concentrations**

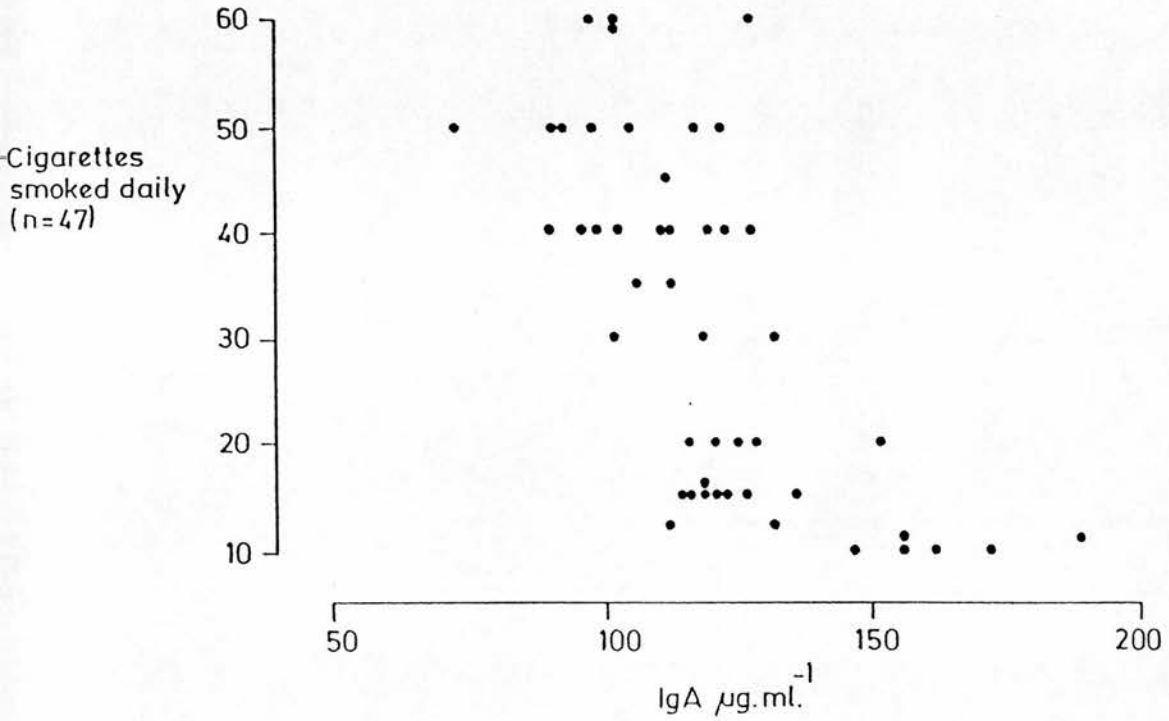


Parotid salivary IgG concentrations in Cairo subjects. Bars show mean ± standard error. No significant differences between groups, (Kruskal-Wallis test).

non-smokers (Figure 9.5). The effects of stopping smoking on salivary IgM concentrations were less consistent. Values were similar to those for non-smokers after 2 years abstinence (p=0.641, Mann-Whitney test, figure 9.6), but at 5 years were significantly higher again than in non-smokers (p=0.0004, Mann-Whitney test).

The findings were identical when the patients with head and neck tumours (the majority being smokers, or recent ex-smokers) were compared with the non-smoking controls (Figures 9.1 - 9.3). Again, salivary IgA concentration

Figure 9.4 **Correlation of cigarette consumption and salivary IgA concentration**



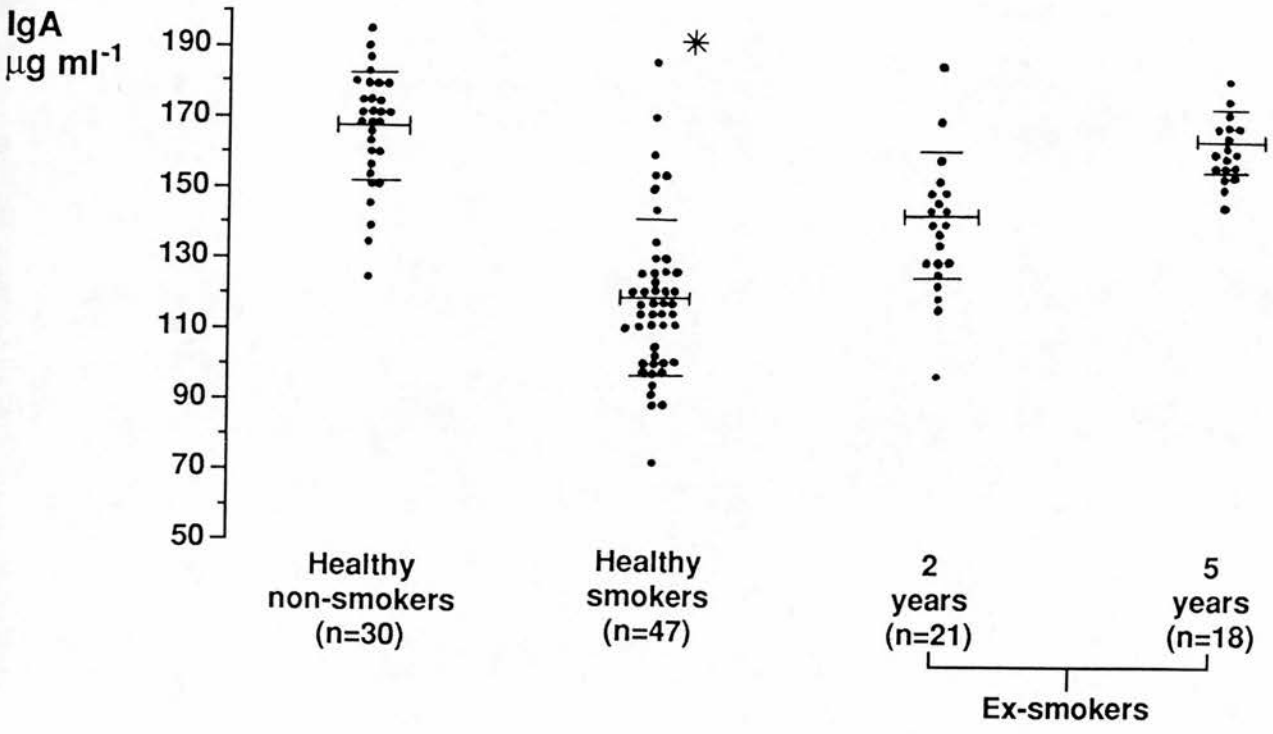
Relationship between daily cigarette consumption and salivary IgA concentration, r_s = -0.68, p<0.00001, (Spearman correlation coefficient).

was markedly decreased and IgM concentration increased, IgG being normal, when compared with values for matched non- smoking controls. No differences were seen between the groups of patients who were untreated, and those who had undergone radiotherapy, (figures 9.1-9.3).

In contrast to the patients with upper aero-alimentary tract tumours, IgM concentrations were just significantly

higher between the group with tumours outwith the upper aero-alimentary tract and age- and sex-matched controls, 28.47 ± 3.55ug/ml vs 26.04 ± 6.3ug/ml (p=0.04, Mann-Whitney test). There were no differences in IgA or IgG concentrations (data not shown).

Figure 9.5 **Salivary IgA concentrations in ex-smokers**



Parotid salivary IgA concentrations in ex-smokers of 20 cigarettes daily, after 2 (p=0.0001), and after 5 (p=0.633) years abstinence compared to healthy non-smokers, (Mann-Whitney test). Current smokers are shown for comparison - *.

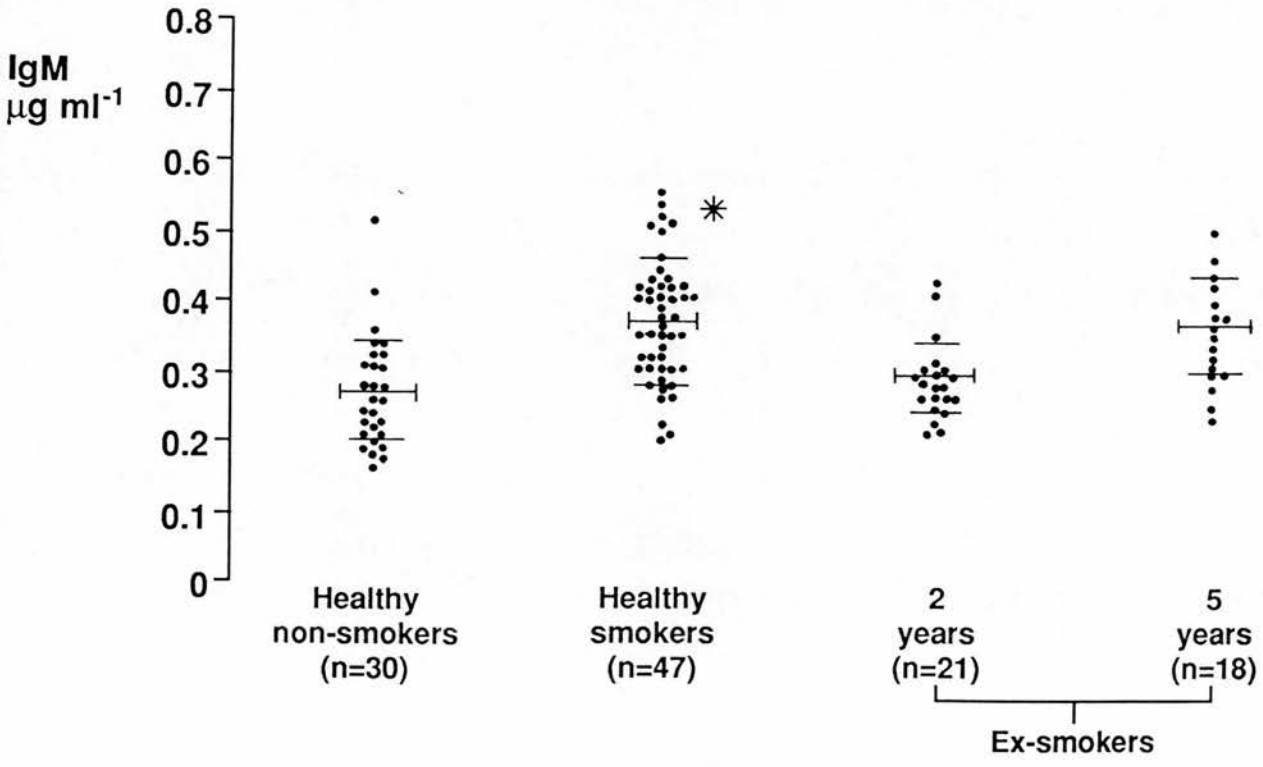
Salivary protein and electrolyte concentrations.

Identical results were found in both centres with no differences in salivary protein and electrolyte concentrations between any of the groups.

Plasma cell numbers in parotid glands

There were no differences in isotype-specific plasma cell numbers in parotid glands when comparing glands from non-smokers with those from smokers, (data not shown).

Figure 9.6 **Salivary IgM concentrations in ex-smokers**



Parotid salivary IgM concentrations in ex-smokers of 20 cigarettes daily, after 2 (p=0.64) and 5 (p=0.0004) years abstinence compared to healthy non-smokers, (Mann-Whitney test). Current smokers are shown for comparison - *.

Conclusions

The influence of smoking on parotid salivary immunoglobulin concentrations was striking, especially when twenty or more cigarettes were smoked daily. Parotid salivary IgA was decreased in a dose-dependent and reversible fashion, whereas IgM was increased. There was no influence on IgG. The findings were independent of alcohol consumption and age. Similarly, salivary IgA was decreased and IgM increased in patients with head and neck tumours, most of whom did or had smoked. There was no consistent effect of radiotherapy. In patients with tumours outwith the upper aero-alimentary tract, there was a marginal increase in parotid salivary IgM concentration.

CHAPTER TEN: INFLAMMATORY BOWEL DISEASE

Introduction and aims

Some in vitro studies on blood and intestinal cells in patients with inflammatory bowel disease suggest that there may be a primary immunodeficiency in the gut that may predispose individuals to these diseases (James et al, 1985; MacDermott et al, 1981; Verspaget et al, 1988). Serum IgA deficiency is also associated with Crohn's disease (Bergman et al, 1973; Soltoft et al, 1972). One of the main aims of this thesis was to determine if there was evidence of mucosal immunodeficiency in patients with inflammatory bowel disease. Two specific questions which were addressed follow on from this main aim; firstly, are there alterations in secretory immunity in parallel with disease activity?, and secondly, are there differences in gut secretory immunity when comparing patients with Crohn's disease to patients with ulcerative colitis?.

Approach

In order to answer these questions, samples of saliva and intestinal lavage fluid were obtained from healthy volunteers and from patients with active and inactive Crohn's disease who were already attending the gastrointestinal unit for investigation by colonoscopy or barium enema. These patients were offered bowel preparation by lavage. The opportunity again arose to study samples of lavage fluid (but not saliva) from the group of patients with inactive ulcerative colitis who had had

lavage for a separate project as detailed in chapter 9. Parotid saliva and intestinal fluid were collected from subjects as previously detailed, and assayed for total immunoglobulin concentrations and IgA, IgM, and IgG antibodies to gliadin, ovalbumin, and ßlactoglobulin.

RESULTS

Saliva.

Total immunoglobulin concentrations were similar in healthy controls and in patients with inactive and active Crohn's disease (table 10.1). IgA and IgG specific antibodies to all three antigens were increased in parotid saliva from patients with inactive and active Crohn's disease compared with healthy subjects (table 10.1).

Lavage.

Total immunoglobulin concentrations were similar in intestinal lavage fluid in healthy volunteers and in patients with inactive Crohn's disease, and in patients with inactive ulcerative colitis. Total immunoglobulin concentrations, of all three isotypes studied, were increased in patients with active Crohn's disease. By far the most striking elevation was of IgG (table 10.2). Specific antibody levels to food antigens in lavage were no different from controls or from patients with inactive or active Crohn's disease. In patients with inactive ulcerative colitis however, IgG antibodies were increased when compared with control subjects (table 10.2).

Table 10.1 **Salivary immunoglobulins and antibodies in inflammatory bowel disease**

	Total Ig	GLI	ßLG	OVA
IgA				
controls	165.8 (66-607)	0.024 (0-0.154)	0.008 (0-0.12)	0.013 (0-0.13)
Crohn's				
inactive	149.4 (24-423)	0.11 * (0.05-9.7)	0.25 * (0.14-0.49)	0.076 # (0.01-0.78)
active	182.2 (69-477)	0.1 * (0.02-0.75)	0.11 * (0.02-0.54)	0.12 * (0.02-1)
IgM				
controls	1.1 (0-556)	0.053 (0-0.3)	0.143 (0.03-0.3)	0.005 (0-0.34)
Crohn's				
inactive	1.12 (0-0.59)	0.048 (0.01-0.11)	0.034 * (0.01-0.09)	0.014 # (0.01-0.31)
active	1.84 (0.44-37)	0.025 (0-0.76)	0.035 * (0-0.17)	0.029 * (0-0.5)
IgG				
controls	3 (0-11.7)	0 (0-0.076)	0 (0-0.029)	0 (0-0.017)
Crohn's				
inactive	1.06 (0.17-11.5)	0.003 * (0-0.024)	0.002 (0-0.016)	0.002 * (0-0.01)
active	1.29 (0.16-34.6)	0.001 * (0-0.038)	0.001 # (0-0.07)	0.002 # (0-0.03)

Total immunoglobulins and antibodies (median + range) in pure parotid saliva from controls (n=22), and patients with inactive (n=7) and active (n=17) Crohn's disease. Key: p = * <0.025, # <0.05, vs controls, (Mann-Whitney test).

Table 10.2 **Lavage immunoglobulins and antibodies in inflammatory bowel disease**

	Total Ig	GLI	ßLG	OVA
IgA				
C	87.1 (10-1042)	0.12 (0-0.885)	0.182 (0-0.6)	0.238 (0-0.76)
CD-I	76.5 (21-272)	0.232 (0-5.6)	0.036 (0-0.14)	0.083 (0.02-0.17)
CD-A	157.8 (23-1256)	0.073 (0-0.38)	0.068 (0.01-0.21)	0.19 (0.06-0.38)
UC	76.3 (7-417) #	0.148 (0-0.93)	0.133 (0-1.03)	0.239 (0.01-0.4)
IgM				
C	5.95 (1.4-35.8)	0.122 (0-1.11)	0.063 (0-0.25)	0.121 (0-0.46)
CD-I	7.29 (0-30.7)	0.021 (0.01-0.11)	0.014 (0-0.05)	0.012 (0-0.22)
CD-A	16.5 (7.1-76)	0.106 (0.02-0.85)	0.026 (0-0.26)	0.03 (0-0.44)
UC	4.6 (1-25.4) *	0.164 (0-0.62)	0.085 (0-0.5)	0.183 (0-0.5)
IgG				
C	0.5 (0-2.2)	0.02 (0-0.06)	0.008 (0-0.1)	0.017 (0-0.1)
CD-I	2.9 (0.5-90.1)	0.004 (0-0.01)	0.001 (0-0.03)	0.002 (0-0.15)
CD-A	36 (2.7-596)	0.031 (0-0.28)	0.007 (0-0.08)	0.014 (0-0.07)
UC	0 (0-19.2) *	0.048 (0-0.11) *	0.044 (0-0.03) #	0.073 (0-0.32) #

Total immunoglobulins and antibodies (median + range) in lavage fluid from controls (C, n=22), patients with inactive (CD-I, n=7) and active (CD-A, n=17) Crohn's disease, and patients with inactive ulcerative colitis (UC, n=30). Key: p = * <0.025, # <0.05, for values comparing all four groups in the column above the symbol (Kruskal- Wallis test).

Conclusions and comments

The generally normal or increased total immunoglobulin concentrations and specific antibody levels, with the odd exception, in both inactive Crohn's disease and inactive ulcerative colitis suggest that there is no secretory immunodeficiency in these two diseases. High total immunoglobulin concentrations in gut lavage fluid in active Crohn's disease may be due to plasma leakage across the inflamed mucosa, or may be derived from the increased numbers of plasma cells present in the inflamed mucosa, or to a combination of both pathologies. Different results for parotid saliva and intestinal lavage in patients with Crohn's disease further supports previous observations in this thesis that parotid saliva does not reflect immune events which occur more distally in the gastrointestinal tract.

There were significant differences in total IgG concentrations in lavage fluid when comparing patients with inactive and active Crohn's disease, but the small number of patients with active disease precludes meaningful correlation of IgG and disease activity.

There do appear to be differences between inactive ulcerative colitis and inactive Crohn's disease, with increased IgG specific antibodies to the food antigens in patients with ulcerative colitis. No comparisons can be made for active disease, since no patients with active ulcerative colitis were studied. There have been few

studies on secretory intestinal immunity and only two of salivary immunity in inflammatory bowel disease, (see chapter 2). These two studies were conducted by the same group, and in whole saliva found an increase of IgA, IgG and IgM in patients with Crohn's disease (Crama-Bohbouth et al, 1984), and an increase in IgA and IgG in ulcerative colitis (Crama-Bohbouth et al, 1989). They noted gingival inflammation in the patients with ulcerative colitis, but no macroscopic differences in the oral mucosa between control subjects and patients with Crohn's disease. In parotid saliva, there were no significant differences in immunoglobulin concentrations in either study, and treatment with corticosteroids decreased immunoglobulin concentrations (although not significantly). The absence of any significant difference in parotid saliva accords with the results from this study.

There are no comparable studies on immunoglobulin concentrations in gut fluid in patients with inflammatory bowel disease. There were no differences in jejunal aspirate immunoglobulin concentrations between a heterogeneous group of patients with Crohn's disease and controls (Jones et al, 1976), but IgG concentration was increased in the freeze-dried faeces of patients with inactive ulcerative colitis (Haneberg and Aarskog, 1975). This latter result is at variance with the data from this study, but the experiments are not really comparable.

CHAPTER ELEVEN: GENERAL DISCUSSION

Introduction

Since there has been no systematic study of gastrointestinal secretory immunity in man, this thesis sought to develop, evaluate, and apply protocols for the study of gut secretions. Saliva was assessed in its own right as a mucosal secretion, and the relationships of saliva to serum, and to gut lavage as a more distal gastrointestinal secretion were also studied. It was hoped that saliva would be representative of more distal gut secretions and could therefore be used as a means of studying the secretory immunity of the gut. This proved not to be the case - in fact there seems to be no easy way to investigate gut immunity.

In addition to the study of healthy volunteers and patients without organic gastrointestinal disease to look at basic gut secretory immunity, groups of patients likely to have abnormalities of gastrointestinal immunity were also assessed. Increased secretory immunity to food protein antigens was indeed present in patients with coeliac disease and dermatitis herpetiformis. Furthermore, study of these patients answered questions on the relationships between antibodies and between immunoglobulins in serum and secretions. During early work, it became apparent that the effects of smoking on secretory immunity especially deserved further study. Finally, to clarify previous reports of immunodeficiency in inflamma-

tory bowel disease, preliminary studies of such patients were undertaken. There was no evidence of secretory immunodeficiency in these studies.

Subjects.

Patients with functional bowel disorders (including those with the irritable bowel syndrome) were used in the thesis as normal control subjects, those with diseases likely to have an immunological component being excluded. Although most clinicians and immunologists do not believe that patients with irritable bowel syndrome have any immune abnormality, some would disagree and cite an immune reaction to food as aetiological. This question was not specifically addressed in this thesis, but is under study at present. For the purposes of this thesis these patients were assumed to be immunologically normal and included in control groups.

Patients with coeliac disease and dermatitis herpetiformis are fairly easy to classify objectively, for example in terms of gut pathology, disease site, or current diet, and form fairly cohesive "experimental" groups. Thus, these patients were well documented for the purposes of this thesis. Unfortunately this cannot be said for patients with inflammatory bowel disease, particularly Crohn's disease. These subjects were much more heterogeneous in terms of disease extent, activity, and treatment (often with immuno-modulatory drugs) than the other groups studied. This part of the thesis is a preliminary

study which lays a foundation for future, extended, enquiry.

In view of the well-documented and profound effects of nutritional status on immunity, future studies of mucosal immunity should include an assessment of nutritional status, and the influence of nutrition and "pure" malnutrition (such as might be found in anorexia nervosa or after extensive small bowel resection for ischaemia) on secretory immunity should be specifically addressed. A whole programme of research is needed to assess this broad and complex issue. One patient group who would be ideal for study are those with coeliac disease. These patients, when newly presenting, have villus atrophy in association with nutritional abnormalities ranging from marked malnutrition to no nutritional deficiency at all. Furthermore, most will improve markedly on a gluten-free diet.

Unfortunately, the limited literature on the effects of nutrition on secretory immunity has concerned heterogeneous groups of subjects, in whom malignancy or chronic infection/infestation may have been the primary problem (Cheatham et al, 1984; Watson et al, 1985 - see review in chapter two). To repeat, studies on homogeneous groups of subjects with pure malnutrition are needed.

Expression of results.

A persistent problem with no easy solution is the question of how best to express antibody levels obtained by

ELISA when there is no weight-based, quantified, standard available. Ideally, isotype-specific antibody should be obtained by passing serum or a secretion known to have high antibody levels over a sepharose gel column or on agarose beads, coated with the specific antigen to allow antibody extraction. The antibody could then be eluted by washing, and a pure protein preparation obtained which could then be used as a standard, and against which test sample antibody levels could be expressed in micrograms, nanograms, or picograms, as appropriate. This exercise is technically feasible, although time-consuming and expensive; if further work is to be carried out using the same antigens and antibodies, it would be valuable to invest time and expense in obtaining the standards.

In the absence of such a standard the best compromise, adopted in this thesis and by many working in secretory immunity, is to express antibody levels as optical density (OD) readings, given as a percentage of the OD reading of a high positive control sample. Multiple aliquots of the high positive control sample are set aside so as to allow valid comparisons between various batches of test samples. A major reason for expressing results as a percentage of a control sample is that optical density readings are not linearly related to specific antibody levels. A further corollary of this is that a comparison between antibodies of differing specificities is not possible. To allow valid comparisons between samples it is important to standardise the assay conditions as much

as possible, since significant variations in results can occur between assays, and preferably one should make comparisons only between samples run in the same batch. Needless to say, a control group should always be used. Using these methods, and means of expressing results, the assays' reproducibility and variation were within acceptable limits, 3.1-13.4% (tables 1 and 2, chapter 4).

The main alternative method of expressing antibody levels would be by diluting out each sample until a standard level of activity (measured spectrophotometrically) had been reached. However this would multiply the number of assays involved, and, since each of the many samples was already being assayed for twelve parameters, this alternative was considered to be impractical.

It is important to state that it is critical to use non-parametric statistical methods when analysing specific antibody levels expressed as OD readings, since, as stated above, OD readings are not linearly related to antibody levels.

Specimen processing and storage.

- Saliva.

Most investigators have found that saliva (especially parotid) is stable when stored frozen, even without any other means of preservation. There may be slight deterioration of proteins over time, but this seems to be of little consequence when a temperature of -70^{oC} is used, and when storage time does not exceed three months. Most assays for this thesis were done within this timespan, and all specimens were stored at -70 ^{oC}. Formal assessment of the effects of storage and freezing were therefore not carried out.

- Lavage fluid.

The necessity of immediate addition of protease inhibitors to gut lavage fluid in order to preserve immunoglobulins was amply demonstrated in this thesis. Again, the fluid was stored at -70^{oC}, and assayed promptly. Therefore no formal study of the effects of prolonged storage was undertaken. The processing and protocol for protease inhibition was taken directly from other investigators, who had given our laboratory their protocol, based on extensive work, some eighteen months prior to publication of the paper (Gaspari et al, 1988). It would be sensible to re-examine the need for each stage of the protocol, which at present uses multiple protease inhibitors and repeated centrifugation, in an attempt to simplify the process. As it stands at present it is somewhat lengthy and complex. It would certainly be worth comparing sim-

pler treatment regimes, with just one or two anti-proteases, for future studies.

Although most subjects found the drinking of the lavage fluid acceptable, many found the taste to be very salty. A reduced sodium preparation is now available, and should be a better alternative, especially if healthy volunteers (with less incentive to comply) are to be involved (Fordtran et al, 1990).

Salivary physiology.

It is obvious from the literature that analysis of mixed saliva is a less than perfect secretion by which to study secretory immunity. Previously, methods of salivary collection have been poor and the simple experiments in this thesis have shown that data on secretory immunity derived from mixed saliva is of questionable value. It is absolutely essential to take account of age and smoking habits. For example, one of my successors, in collaboration with me, studied an aged population. Despite contradictory literature on whole saliva, there was not a single paper on pure parotid saliva, and we found a striking effect of age on salivary immunity, by using simple, straight-forward, methodology.

The original premise for this thesis was that food, time, and alcohol would have to be controlled for when collecting saliva, because of their likely influence on salivary immunoglobulin and antibody levels. In fact meals were

the only consistent influence, and then only transiently; thus the only absolute recommendation for future work would be to sample after a short fast. There was a marked variability in the concentrations of immunoglobulin and levels of specific antibody over the 18 hour period studied. The fall in these parameters after meals might be explained by acinar or cellular depletion consequent on the high salivary flow which occurs during eating (a washout effect), levels rising slowly after eating, until the next meal. The high protein concentration in saliva after food may be due to induction of digestive enzyme synthesis and release upon eating. The disparity between the fall in immune parameters and the increase in total protein may be due to a differential capacity for synthesis or storage, or, if digestive enzymes were responsible for the increased protein, that immunoglobulin release is not stimulated by eating, whereas enzymes are. There are probably many various influences on these parameters; the only one definitely identifiable was, as stated above, that of eating a meal, particularly breakfast. The best solution seemed to be to take samples after an overnight fast, or at least one hour after a meal, to permit comparison of the results.

I had proposed that a large dose of oral alcohol would lead to gut damage and an increase in gut permeability, which would in turn eventually stimulate B cells to produce more antibody, this being measured as a change in salivary antibody levels. However, the effect of a large

acute dose of alcohol on salivary antibody levels was inconsistent although wide fluctuations in antibody levels were observed, more so than in the 18 hour study. Both smokers and non-smokers were included, since the influence of smoking had not been appreciated at the time, and this heterogeneity may have confounded interpretation of the results. The sugar permeability test only detected a change in gut permeability in one non-smoking volunteer, whose antibody levels rose dramatically. The permeability test may not have been sensitive enough to detect more subtle changes in mucosal permeability in the other subjects, as the size of the molecules used limits the range of sensitivity of the investigation. However, large doses of alcohol were used, and any real and physiologically significant alteration in salivary immunity seems unlikely. It would be valuable to assess the acute and chronic effects of alcohol on intestinal secretions, especially in those with alcohol-related diarrhoea.

Lavage physiology.

For the purposes of this thesis, the first clear samples of effluent obtained at gut lavage were taken to be representative of lavage. Indeed the serial study of two consecutive samples found that a steady state, in terms of IgA concentration, was reached in lavage fluid once the fluid had become clear. This work is at present being repeated and extended to include several serial samples. In the future, repeat lavages need to be performed on

several days, and after longer periods of time to assess the physiological dynamics of the fluid and the reproducibility of the technique. Lavages could also be performed prior to, and after excluding specific antigens from the diet for a period of time. Similarly, subjects taking an elemental diet could be investigated to determine whether or not gut fluid, like parotid saliva, maintains antibody levels in the absence of dietary antigen stimulation.

Kinetics of antibody levels.

Specific questions concerning the influence of dietary antigen on levels of salivary antibodies were addressed using a variety of approaches. Healthy patients were divided into those including and those excluding bread or eggs or milk in their diet. Groups of patients with dermatitis herpetiformis both on a normal, gluten- containing, and a gluten-free diet were studied. Finally, a heterogeneous group of patients begun on an elemental diet were assessed. Generally, antibody levels to dietary antigens were similar, irrespective of either dietary antigen intake or mucosal integrity. This suggests that, at least for food protein antigens, salivary antibody levels are maintained in the absence of dietary antigen stimulation for at least one year.

There are a number of points that need to be kept in mind when interpreting these results. Firstly, avoidance of certain dietary antigens may not be complete. Secondly, only the study of subjects taking an elemental diet was prospective, bringing recall bias into account for the

retrospective studies. Thirdly, the prospective study of subjects on elemental diet was heterogeneous in terms of disease, concomitant therapy, and duration of diet. However, despite these qualifications, the uniformity of results suggest that the findings are valid.

Animal studies of antigen-stimulated mucosal antibody production suggest that the quantity and number of doses are critical determinants of not only the magnitude but also of the quality of response. It must be remembered that these experiments were conducted in genetically identical, immunologically naive, animals and it may be inappropriate to extrapolate the results to man. The kinetics of antibody production have not been studied in this thesis, nor have non-protein antigens. Experiments with other antigens of different origin and of antigens entirely novel to subjects would give valuable information. It may be that quite different patterns of antibody levels would result if, for example, bacterial antigens (associated with highly immunogenic lipopolysaccharides) were used to stimulate a response. Certainly, other antigens are as relevant as food antigens in terms of disease.

Coeliac disease and dermatitis herpetiformis.

Other groups have been assaying saliva for anti-gliadin antibody levels as a test for coeliac disease. These studies have made two suppositions; firstly they have assumed that salivary antibodies reflect distal gut

antibodies, and secondly that anti-gliadin antibody levels in distal gut secretions can distinguish between healthy subjects and patients with coeliac disease. Unfortunately neither of these suppositions have been either thoroughly tested nor proven.

One of the main aims of this thesis was to determine if the first hypothesis was correct and that salivary antibodies and immunoglobulins did reflect distal gut secretions. To accomplish this, the results from gut lavage were taken as the "gold standard". Then, antibody levels and immunoglobulin concentrations from parotid saliva were compared with those obtained from lavage. Data from saliva generally did not correlate with that from gut lavage, nor did it correlate with data obtained from jejunal aspirate.

The study of parotid saliva from coeliac patients also allowed comparison with control subjects to see if these groups could be separated by measuring antibody levels in saliva or gut lavage. If this had been the case, salivary antibody levels or immunoglobulin concentrations might have been of use in diagnosis or follow-up of patients with coeliac disease. Finally, samples of secretions from these patients permitted an assessment of the effects of mucosal damage on antibody levels to dietary antigens.

Although salivary antibody levels were higher in coeliac disease than in control subjects, the degree of overlap

between the groups prevents saliva from being used as a diagnostic marker. The persistence of salivary antibody levels in patients with coeliac disease and dermatitis herpetiformis on a gluten-free diet, even when complete mucosal healing has occured, also precludes the use of saliva for monitoring dietary adherence, mucosal healing, and relapse in these conditions.

It is not apparent why salivary antibody levels in dermatitis herpetiformis are similar whatever the state of the mucosa. It might be proposed that if there has never been an enteropathy present that antibody levels would be low but this did not appear to be the case. Transient previous abnormality cannot be excluded, and sensitive histological techniques (for example quantitative morphometry, intra-epithelial lymphocyte counts, and crypt cell mitotic rates) were not employed to detect subtle forms of enteropathy. However other work from this laboratory supports an abnormality of secretory immunity in dermatitis herpetiformis, independent of any enteropathy (O'Mahony et al, 1990). It is of interest that, despite the fact that gliadin is the "toxic" antigen, there was strong positive correlation between antibody levels to all antigens, even irrespective of isotype. This suggests that the mucosal antibodies are produced as part of a generalised secretory response, secondary to a primary pathological insult, a conclusion also supported by the above study.

In gut lavage there were not only significant differences in antibody levels to gliadin but also to ovalbumin and ßlactoglobulin between controls and patients with both treated and untreated coeliac disease. These differences were not present in parotid saliva. In addition, total IgM and IgG concentrations were increased in the lavage fluid of untreated coeliac patients. Interestingly increased numbers of IgM and IgG plasma cells have been reported in the jejunal mucosa of patients with coeliac disease (Lancaster-Smith et al, 1974; Scott et al, 1984). It might be reasonable to attribute these high antibody and immunoglobulin levels to increased secretory immunity in an area of inflamed and damaged gut mucosa. The increased IgG in untreated disease might be explained by plasma leakage from the inflamed mucosa although it must be said that IgA is not proportionately increased as might be expected, and an increase in mucosal IgG plasma cells has previously been demonstrated, (Lancaster-Smith et al, 1974; Wood et al, 1987), suggesting that a "leak" may be too simplistic an explanation and that a local increase in production may be present. Furthermore, although in treated patients the lavage IgG concentration was not different from controls, IgM concentrations remained high.

Other work of great interest from our laboratory, referred to above, found that jejunal aspirate total IgM concentrations and IgM antibodies to gliadin, ovalbumin, and ßlactoglobulin are high not only in patients with

untreated coeliac disease, but also in patients with dermatitis herpetiformis with a normal jejunal mucosa, as assessed by standard histology, intra-epithelial lymphocyte counts, and quantitative morphometry. Jejunal aspirate total IgA and IgG concentrations were also increased, but not as markedly as IgM (O'Mahony et al, 1990). A two stage model of coeliac disease is proposed, in which inappropriate gluten sensitivity is quite common, genetically restricted, and expressed in the gut (and also in the kidneys and skin) via T-cell mediated immunity as a spectrum of functional and histological abnormalities. Expression of the full intestinal lesion is dependent on a second factor (of which a number are proposed) to drive the enteropathy from latent to overt.

Relationships between antibody levels in various fluids

Data from both animal work and human studies has demonstrated the existence of a common mucosal immune system. Thus it be expected that one mucosally derived secretory fluid would be representative of another. A major aim of this thesis was to define whether or not this was the case. At the start of this thesis I knew from the literature that antibodies to mouth commensal bacteria were detectable in parotid saliva (Gregory and Allansmith, 1987; Smith and Taubman, 1987), and that that specific antibodies to gut pathogens (and other orally administered bacteria) could be found in saliva either after natural infection or immunisation (Clancy et al, 1983; Carlsson et al, 1985; Jertborn et al, 1986). It was also

apparent that there was generally little or no salivary antibody response after parenteral immunisation (Baumann et al, 1985; Waldman et al, 1986). Additionally, from the rare previous studies addressing the question, there did not seem to be any clear relationships between antibodies induced in both saliva and intestinal fluid by gut infection (Jertborn et al, 1986; Stoll et al 1986), nor had anyone described salivary immune responses to either a primary systemic or a localised, non-mucosal, infection. In the thesis I sought to formally study these points.

The results from my experiments did not substantiate the hypothesis that one mucosally derived secretory fluid was representative of another. With respect to comparisons between serum and saliva, current theory suggests that there is either an inverse or no relationship between antibody levels. The findings of this thesis suggest mainly no relationship, but when one is present the correlations are direct and positive. This was true for both antibodies of the same isotype and also for serum IgG and salivary IgA antibodies. In fact, the positive correlations between salivary IgA and serum IgG antibodies in patients with coeliac disease and dermatitis herpetiformis suggest activation of both the salivary and systemic immune systems in parallel.

Furthermore, although parotid saliva differs from jejunal aspirate, both in the way it encounters antigen and secretes fluid into the gut (via a duct), it is unlikely

that these differences wholly account for the lack of correlation between saliva and aspirate, since, despite the fact that the data is taken from an heterogeneous group of subjects, antibody titres in aspirate and gut lavage did not correlate either. One explanation for this may be differences in local antigen concentrations.

Thus, relationships between immunoglobulins or specific antibodies in mucosal secretions were rare, and most of the significant correlations were between immunoglobulins or antibodies in serum and in a secretion. Direct correlations were most often found when mucosal damage was, or previously had been, present. This suggests that when the mucosal barrier is breached, loss of tolerance may occur, and both the systemic and mucosal immune system may be stimulated to a greater or lesser extent.

My further studies since the completion of this thesis have demonstrated a five to ten-fold rise in both total and secretory IgA and in specific antibody levels to Streptococcus mutans species (pathogenic oral bacteria) following tonsillitis and peri-tonsillar abscess. Therefore, although the parotid glands have been shown to be part of the common mucosal immune system they may also reflect the systemic immune response, and, taken all together, my work suggests that the parotids are an area of interface between the two systems. After all, unlike the majority of the mucosal associated lymphoid tissue the parotid glands have not been shown to take up anti-

gens directly from the exterior, and may not even encounter antigen directly at all, the stimulus to antibody production possibly arising from circulating lymphocytes which have migrated from an area of antigen challenge.

What are the likely mechanisms involved in parotid salivary antibody production?. It is unlikely to be local antigen leading to direct stimulation, or to plasma leak into parotid saliva. It is most likely to be circulating B cells, from the gut or oral mucosa, or from local lymph nodes and bone marrow in the case of systemic infection which gives rise to the specific salivary antibody response. Migration of T cells leading to B cell maturation and activation could also be involved, as could T cell signals, either directly or via cytokines. Studies of circulating lymphocytes and parotid salivary cytokines would be two ways of investigating the mechanism.

So lavage fluid has been extensively investigated in many subjects, along with parotid saliva. In fact, the assumption that saliva is a good representative of mucosal secretions is not borne out either by this thesis or by the current literature. Naively at the beginning of this thesis I had hoped salivary immunity would be proportional to distal gut immunity, and that I would be able to use saliva to investigate important questions relating to distal gut immunity and to oral tolerance. However, although there were significant differences both in salivary and in lavage antibody levels between groups of

well-characterised patients with coeliac disease, and although patterns of antibody levels for these groups in saliva often parallelled those in gut lavage, for an individual saliva could not be used to predict gut lavage antibody levels.

Smoking and mucosal immunity.

Much work has been conducted on systemic immune status but, surprisingly, the influence of smoking on mucosal immunity has been relatively neglected. Smoking is strongly associated with epithelial tumours of the upper oro-gastro-intestinal tract (Kleinsasser, 1983). The incidence of ulcerative colitis is much lower in smokers than in non-smokers or ex-smokers (Tobin et al, 1987; Boyko et al, 1987; Vessey et al, 1986). A recent report that smokers have reduced numbers of antigen-presenting cells in the cervical epithelium is of interest (Barton et al, 1988).

A striking and reproducible influence of cigarette smoking on salivary immunoglobulins was demonstrated in these investigations. In healthy smokers, the concentration of IgA in pure parotid saliva was reduced, and that of IgM increased, when compared with non-smokers. For IgA, this effect was strongly dose dependent, and reversible; salivary IgA concentrations were similar to those for non- smokers in a group of healthy subjects who had stopping smoking at least five years before. Differences in parotid salivary immunoglobulin concentrations between

healthy non- smokers and patients with head and neck cancer might be attributed solely to the smoking habits of the latter. Such a relationship may be blurred by the fact that many smokers are heavy alcohol drinkers (Flamant et al, 1964), and alcohol consumption may increase serum (and therefore possibly salivary) IgA levels (Sherlock, 1989). Alcohol is an independent risk factor for squamous cell cancer of the head and neck (Broitman et al, 1983). This complicating factor of alcohol was avoided by using non-drinkers in the second, case-controlled phase of the study in which identical results were obtained.

The similarity of the findings between healthy smokers and patients with epithelial head and neck tumours probably reflects the fact that most people who develop these neoplasms smoke. Although many patients in the untreated tumour group and in the post-radiotherapy group had stopped smoking at the time of sampling, most commonly at the time of diagnosis, sufficient time may not have elapsed to allow any potential normalisation of salivary immunoglobulin levels. However, the majority of patients in the prospectively studied group from Edinburgh undergoing radiotherapy said that they had never smoked. It may be that the salivary immunoglobulin alterations seen are a secondary phenomenon, reflecting immune disturbance secondary to tumour. The negative findings in the patients with tumour elsewhere would suggest, if that were the case, that there must also be a local component to

this. An attractive alternative explanation is that mucosal immunodeficiency may predispose to tumour formation, as others have proposed (Kleinsasser, 1983).

I am aware of four previous reports of salivary immunoglobulins in smokers (Watanabe et al, 1983; Hersey et al, 1983; Bennett and Reade, 1982; Olson et al, 1985), relating to small numbers of selected patients, and using whole saliva. Even in the absence of oral sepsis or obvious radiation damage, contamination with crevicular fluid is likely to influence results. This is highlighted by the discrepant results in the literature on the influence of smoking on immunoglobulin content of mixed saliva. Bennett and Reade found that salivary IgA concentration was low in 8 elderly, edentulous male smokers, in contrast to Watanabe et al who reported high mixed salivary IgA levels in a group of smokers and drinkers, and in head and neck cancer patients. Hersey et al reported a striking decrease in salivary IgG (probably derived from crevicular fluid or inflammatory exudates) in a small group of healthy smokers who stopped smoking. Olson et al found no change in whole salivary IgA when smokers stopped smoking and took nicotine or placebo chewing gum. The use of pure parotid saliva for assay is therefore particularly important in patients with head and neck tumours and after radiotherapy, when there may be immunosuppression and oropharyngeal superinfection.

Studies of serum and circulating leucocytes have been

used to determine the effects of smoking on systemic immunity. Smokers have increased polymorphonuclear neutrophil counts, decreased natural killer cell activity, an increase in total T cell numbers with a decrease in the $T_{helper/suppressor}$ cell ratio in heavy smokers (reviewed by Holt, 1987). The decrease in T_h cells in both blood and broncho-alveolar lavage has been noted by more than one group, and shown to be reversible on stopping smoking (Costabel et al, 1986; Miller et al, 1982). In smokers, serum IgA, IgG, and IgM are generally lower by 10-20% than in non-smokers (Gerrard et al, 1980; Holt, 1987), with higher levels of serum IgD and IgE in smokers (Holt, 1987). Smokers have a generally decreased systemic response to antigens encountered at the nasal and respiratory mucosae (Andersen et al, 1982; McSharry et al, 1985), although at the mucosal level conflicting results have been reported for total IgA in broncho-alveolar lavage fluid, with a decrease (Gotoh et al, 1983) and a three to four-fold increase (Velluti et al, 1983) reported; at the nasal mucosa a decrease in local antibody levels in smokers is reported (Rylander et al, 1982). In a group of patients with head and neck tumours high whole salivary IgA levels were shown to correlate with tumour load, and decrease towards normal after successful treatment (Brown et al, 1975).

The increase in IgM levels might be interpreted as a compensatory rise. This concept is a little naive; IgM has very different properties to IgA, and its higher

concentrations may reflect immunopathology rather than simple compensation. It is probably unnecessary to confirm the self reported cigarette consumption; there is no reason to suppose that any errors in reporting would vary between light, moderate or heavy smokers, and others have shown self-reported smoking habits to be reliable (Williams and Gillies, 1984).

Although no statistically significant difference between smokers and non-smokers was found in gut lavage fluid It may be that, due to too many light smokers and too few subjects, a type II error has occurred. A larger study of heavier smokers of more than twenty cigarettes daily should be undertaken to clarify the issue. Although no consistent effect of alcohol was demonstrated on parotid salivary immunity it would be sensible to exclude heavy drinkers from such a study.

Unfortunately, there were too few patients with inactive and active Crohn's disease to assess the influence of smoking on mucosal immunity in this condition. This would be of great interest, given the association of smoking with Crohn's disease, the findings of the thesis, and postulated immunodeficiency present in these patients (MacDermott et al, 1986; Verspaget et al, 1988).

Thus it has been demonstrated that smokers have a dose-dependent and probably reversible humoral mucosal immunodeficiency, as reflected either directly or otherwise by

salivary IgA concentrations. Studies of mucosal immunity at other surfaces are necessary and should include investigation of T cell factors and other regulatory mechanisms. It would be valuable to assess the effect of smoking on the quality and magnitude of the mucosal response to novel antigens. It would also be valuable to use bacterial antigens, and not to confine the work to dietary protein antigens as previously, since the former may be more directly relevant to gastrointestinal pathology. If changes in secretory immunoglobulins reflect abnormalities of other, as yet unexplored components of mucosal immunity, new avenues of pathogenesis of smoking-related diseases may emerge.

Inflammatory bowel disease.

Information on immunity in patients with inflammatory bowel disease is becoming more and more important as the numbers of patients increase and as the high morbidity becomes apparent, especially in the young (Barton et al, 1989; Barton and Ferguson 1990). I had proposed that there might be a primary mucosal secretory immunodeficinecy which could be quite localised and very specific. My studies in this thesis were only the early phase of a larger study on this topic, but certainly found no evidence of mucosal secretory immunodeficiency, although others had previously suggested that immunoglobulin production by lamina propria lymphocytes was decreased (see chapter two; also - MacDermott et al, 1981; Verspaget et al, 1988). In fact in parotid saliva there were

generally increased levels of antibodies to food protein antigens, with no alteration in total immunoglobulin concentrations.

Although a different pattern of antibodies and immunoglobulin was apparent in lavage in patients with inflammatory bowel disease, again there was no evidence of immunodeficiency. Total immunoglobulin concentrations were increased in fact, and strikingly so in respect of IgG, with no difference in specific antibody levels. Again this increase might be explicable on the basis of a plasma leak, and further work since the completion of this thesis by co-workers in this laboratory suggests that this is the case (O'Mahony, personal communication). However others have shown high numbers of IgG plasma cells in the gut of Crohn's patients (Brandtzaeg and Baklien 1976; Bookman and Bull, 1979; Kett et al, 1987), and indeed increased synthesis of IgG (Bookman and Bull, 1979; McClelland et al, 1976; Verspaget et al 1988), although this has been disputed (MacDermott et al, 1981) and qualitative differences in IgG subclass distribution have been shown (Kett et al, 1987; Scott et al, 1986).

The markedly elevated lavage fluid IgG concentrations have been confined to patients with active Crohn's disease, and seem to be a marker of early inflammatory activity when other features such as radiographic studies are normal. As such, lavage fluid IgG concentrations are now being used in clinical situations in this hospital.

Secretory immunodeficiency predisposing to or as a result of inflammatory bowel disease has not been excluded by the results from these studies however. For example, others have suggested that IgA subclasses may differ in these patients (MacDermott et al, 1986). This was not addressed in this thesis.

Further study of secretory immunity in inflammatory bowel disease would be of value. It would be important to assess the secretory immune response to novel antigens in dynamic studies. It would also be valuable to use bacterial antigens, and not to confine the work to dietary protein antigens as previously, since the former may be more directly relevant to gastrointestinal pathology.

SUMMARY

New approaches to the investigation of mucosal humoral immunity in man have been explored. Pure parotid saliva, jejunal juice, and intestinal fluid obtained by whole gut lavage have been used to describe some areas of basic physiology of secretory fluids. Comparison of serum, parotid saliva, jejunal aspirate, and intestinal fluid immunoglobulin concentrations and specific antibody levels has allowed correlations between these fluids to be made and their relationships determined. Investigation of patients with coeliac disease and dermatitis herpetiformis has shown that, as in serum, there is up-regulation of secretory immunity. A striking influence of

smoking on salivary immunity has been established. Finally, no evidence of mucosal immunodeficiency in patients with inflammatory bowel disease has been demonstrated.

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(i) Pierce protein assay.

A standard protein solution of known concentration (2mg/ml) was made up with bovine serum albumin in distilled water, and serial dilutions of this solution were made to give standards with a range of protein concentrations from 0.2 - 1.2 mg/ml. Pierce reagent A (sodium carbonate, sodium bicarbonate, bichinchoninic acid [BCA], and sodium tartrate, in 0.1N sodium hydroxide) were added to reagent B (4% copper sulphate.$5H_2O$) in a ration of 50:1. One tenth of a millilitre of the diluted standard or test solution (saliva) was pipetted into a test tube. Carbonate buffer was used as a blank reference. Then, 2.0 mls of Pierce working reagent (A + B) were added to each tube for testing and to the blank, and mixed well. The tubes were incubated at 37^{oC} for 30 minutes then cooled to room temperature. The absorbance of the samples at 562nm was then read against the blank on a standard spectrophotometer (Dynatech Microelisa Autoreader M580), subtracting the value of the blank. The known standard dilutions were then plotted against absorbance, and standard curves constructed to calculate the protein concentration of each test sample. Serial dilution curves were constructed to determine the optimum dilution of the test samples of saliva.

(ii) ELISA for serum antibodies to food proteins.

ELISA plates (Microelisa M129B, Dynatech, Billingshurst, Sussex) were coated with 5 ug/ml of antigen (gliadin or ovalbumin or BLG) dissolved in carbonate buffer at pH 9.6, using 125 ul to coat each well. The coated plates were incubated overnight at 4^{oC}, then washed three times with 0.9% NaCl with 0.05% Tween (Sigma chemicals ltd., Poole, Dorset), and blocked with 0.9% NaCl with 0.05% Tween 20 and 1% adult bovine serum (Scottish antibody production unit, Carluke, Lanarkshire). To measure antibodies in serum, serum was diluted 1:200 to measure IgG antibodies, and 1:100 to measure antibodies of the IgM and IgA isotype.

To each well in duplicate pairs, 125 ul of diluted sample was added, using a high positive serum as a control, and the plates were again incubated overnight at 4^{oC}. After incubation, the plates were washed with 0.9% NaCl with 0.05% Tween , and then blocked with 0.9% NaCl with 0.05% Tween 20 and 1% adult bovine serum. Following this, each well was treated with 125 ul of 1/5000 affinity-purified, alkaline phosphatase-conjugated, goat anti-human IgA, IgG, or IgM (Northeast biomedical laboratories, Uxbridge, Middlesex) diluted in blocking solution. The plates were washed with 0.9% NaCl with 0.05% Tween , and blocked with 0.9% NaCl with 0.05% Tween 20 and 1% adult bovine serum before the addition of 1mg/ml of paranitrophenylphosphate (PNP) substrate (104-105, Sigma) in 10% diethanolamine (DEA) buffer. The resultant colour reaction was read on ELISA reader at OD 405 nm, allowing the OD value of the high positive control to develop to 1.000. The arithmetic

mean of the OD reading of each paired test sample was expressed as a percentage of the OD reading of the high positive control.

(iii) Immunoperoxidase staining of parotid glands.
The parotid gland specimens obtained at post-mortem or per- operatively were fixed for a minimum of 24 hours in 10% buffered formal saline. Using a Histokinette machine the specimens were immersed in increasing concentrations of alcohol from 70% upto 100%, followed by chloroform and then paraffin wax, in which they were mounted. The mounted specimens were cooled on ice to reduce paraffin wax crystallisation and stored dry until processing. Prior to processing the blocks were once more cooled on ice and sections 3 um thick were cut on a microtome, slides being previously coated with poly-L-lysine 0.01% to assist adhesion. Sections were dried in an oven at 37^{oC} for 20 minutes and were then ready for staining.

For staining, sections were de-waxed by immersion in Histoclear for five minutes on two occasions. Dehydration of the specimen was accomplished by soaking in absolute alcohol (IMS 99) for 5 minutes on two occasions. After this they were treated with 1% hydrogen peroxide in methanol for 20 minutes to block endogenous peroxidase, immersed in tap water once and in tris buffered saline (TBS) at pH7.6 twice , for 5 minutes each stage. After a soak in normal donkey serum with TBS for 10 minutes to help prevent non- specific binding, the areas around the specimens were drained and dried, and the primary antibody was applied for 60 minutes. To detect IgA-positive cells for example, sheep IgA anti-human IgA was used.

This was followed by rinse and then two 5 minute immersions in TBS, after which the secondary antibody (e.g. donkey IgG anti-sheep IgA) was applied for 60 minutes. A further rinse and two 5 minute immersions with TBS were followed by coating the specimens for another 60 minutes with sheep peroxidase-antiperoxidase to combine with the anti-sheep antibody, after which they were rinsed and immersed in TBS in a similar manner to previously. Diaminobenzidine was added for 5-10 minutes to develop the brown/black colour reaction, then the specimens were soaked in TBS followed by tap water, each for 5 minutes, before treatment with haematoxylin for 5 minutes to stain up cell nuclei. Soaking in tap water was followed by a 10 second immersion in 1% acid alcohol to remove excess haematoxylin, and then another 2 minute rinse with tap water followed before "blue" in lithium carbonate was added which turned the haematoxylin from red to blue and in doing so stabilised the reaction.

The specimens were then washed in tap water for 5 minutes prior to dehydration for three 30 second periods in 70% alcohol, absolute alcohol, and finally isopropanol. Treatment with Histoclear for 30 seconds, followed by immersion for 5 minutes raised the refractive index and

improved resolution. Finally the sections were mounted in the synthetic resin DPX (RA Lamb, North Acton, London). A positively stained cell appeared brown or black, and the nuclei blue.

(iv) Computing hard- and software.

Table

Hardware
Amstrad PCW 8512
DCS PCII ten
DCS ST 15
IBM PS/2 30 286
Hewlett-Packard laserjet III printer

Software
Amstat 1, 1g, 2a-d statistical programmes
dBASE III Plus data storage, retrieval, and sorting
Minitab statistical programme
Wordstar version 4.1 word-processing
Dynatech EIA-soft ELISA interpretation

The Amstrad PCW8512 and Amstat statistical programmes provided all the necessary analyses, but were limited in terms of flexibility. Data entered onto a storage and retrieval system could not be transferred to the statistics programme and had to be re-entered. Once entered, the data points were not available for the necessary manipulations without substantial re-entry of much of the data again. The system was very slow at calculation, and frequently "dumped" large columns of data, when the limited memory was exceeded. The system could not safely handle more than 10 columns approximately 40 points long.

Minitab on the other hand could import data, with some awkward manipulation, from dBASE III Plus and rapidly analyse large files of data with ease. Again, manipulation was needed to obtain paired t-tests, and non-parametric correlation and significance values.

Initial data storage and statistical analysis was done on the Amstrad computer using the Amstat statistical software programmes. It was soon apparent that the limited flexibility and capacity of this system could not handle the necessary tasks. Fortunately the IBM and IBM-compatible DCS machines, along with dBASE III Plus and Minitab became available. They offered high capacity data storage, flexibility and selectivity of data access, and the necessary RAM to rapidly perform all the tasks required, and were used thereafter.

(v). ELISA for total salivary immunoglobulins - IgA, IgG, IgM, and for secretory IgA

Standards.

Colostral IgA (Sigma) and human serum immunoglobulin (SPS 101, Human Protein Reference Unit, Royal Hallamshire Hospital, Sheffield) were used as standards. The initial dilution of the secretory IgA standard was 0.5 ug/ml, whilst for the IgG and IgM standards, the initial dilution was 3 ug/ml.

Method.

Immulon I ELISA plates (Microelisa M129A, Dynatech) were coated with a 1/5000 dilution of affinity-purified goat anti-human immunoglobulin Fc alpha or gamma or mu antibody (Northeast Biomedicals) in 0.1 M carbonate buffer, pre- diluted 1:1 with distilled water, at pH 9.6-8, using 125 ul per well. The coated plates were incubated overnight at 4°C, and then washed three times with 0.05% Polyoxyethylene sorbitan monolaurate (Tween 20) in 0.9% NaCl, and blocked with 1% adult bovine serum diluted in the same. Saliva was diluted 1:10, and then double diluted down to 1/100 to measure IgG and IgM; to measure IgA the initial dilution was 1:100, followed by double dilutions down to 1/6400. All dilutions were made with blocking agent, and 125 ul of each diluted sample were added to wells in duplicate.

The plates were incubated overnight at 4°C, then washed, and treated with 125 ul of 1/5000 affinity-purified, alkaline phosphatase-conjugated, goat anti-human IgA, IgG, or IgM. (Northeast biomedicals) in blocking solution, and washed again. Then 1mg/ml of PNP substrate (104-105, Sigma) in 10% DEA buffer was added, and the subsequent colour reaction read on an ELISA reader at OD 405 nm. The OD of the highest concentration of the standard was allowed to develop to give an OD reading of 1.000 before the plate was read. Immunoglobulin concentrations in the test samples were calculated from the linear portion of the test sample curves compared to a parallel part of the standard curve.

(vi) ELISA for isotype-specific salivary antibodies to gliadin, ovalbumin, and ßlactoglobulin

ELISA plates (Microelisa M129B, Dynatech) with 5 ug/ml of antigen (gliadin, or ovalbumin, or BLG) dissolved in carbonate buffer at pH 9.6, using 125 ul of solution per well, and incubated overnight at 4°C. After this, the plates were washed three times with 0.9% NaCl with 0.05% Tween, and blocked with 0.9% NaCl with 0.05% Tween 20 and 1% adult bovine serum. To measure antibodies in saliva, the saliva was diluted 1:2 with diluent to measure IgG and IgM, and diluted 1:5 to measure IgA. To each well in duplicate was added 125 ul of diluted sample, using a high positive serum sample as a control, and the plates were incubated overnight at 4°C. After washing again, plates were treated with 125 ul of 1/5000 affinity-purified, alkaline phosphatase-conjugated, goat anti-human IgA, IgG, or IgM (Northeast biomedicals) diluted in

blocking solution, and were once more washed. PNP substrate at 1mg/ml was added (104-105, Sigma) in 10% DEA buffer, and the subsequent colour reaction read on an ELISA reader at OD 405 nm, allowing the OD of the control sample to develop to 1.000. The arithmetic mean of each test sample OD reading was expressed as a percentage of the control value.

(vii). ELISA methodologies for lavage fluid
The ELISA protocols for measurement of total immunoglobulins and antibodies to foods in lavage fluid were the same as for saliva, except for the dilution of the standards when measuring total immunoglobulin concentration. For lavage this was as follows - SPS 101 standard human immunoglobulin reagent for IgM and IgG was initially diluted to a concentration of 3 ug/ml for each immunoglobulin, and followed by doubling dilutions.

(viii) Standards for ELISA
Initially the British working reagent for human serum immunoglobulins IgG, IgA, and IgM 67/99 (National Institute for biological standards, Potters Bar, Hertfordshire), based on the World Health Organisation 1st international reference preparation, was used as a standard for all total immunoglobulin assays. Subsequently SPS 101 (Protein Reference Unit, Royal Hallamshire Hospital, Sheffield) was used as a colostral standard. For total immunoglobulin assays, the standard was double diluted out, beginning at an initial dilution of 1.25ug/ml for optimum assay conditions. For secretory IgA, the optimum initial dilution was found to be 0.5ug/ml. Test samples were also double diluted out, and the concentration of immunoglobulin determined by comparing the curve of OD readings to the standard curve, finding the parallel values, and taking their arithmetic mean. This could be performed automatically, but less reliably, by computer using the Dynatech "EIA-soft" programme.

(ix) **Clinical Questionnaire**
1.

Hospital no._____ sample no._____ Study no._____

date__/__/__ serum no._____ sex____ age____

Surname_____ name_____

Address_____ tel._____

diagnosis_____

drugs_____

alcohol_____units/wk Smoker_____/day dentures_____

oral sepsis____ Y/N time of sample _____

diet- contains: eggs_____ bread_____ milk_____ Y/N

Vol.___mls. Pr.____mg/ml Cell./Man.____ Jej.Bx_____

SERUM RESULTS

....

SALIVA RESULTS

....

LAVAGE RESULTS

....

ASPIRATE RESULTS

....

(x) GLOSSARY OF ABBREVIATIONS, TERMS AND SYMBOLS

ßLG	beta-lactoglobulin
BSA	Bovine serum albumin
C. albicans	Candida albicans
DEA	diethanolamine
DTT	dithiothreitol
E. coli	Escherichia coli
EDTA	ethylenediaminetetracetic acid
ELISA	enzyme-linked immunosorbent assay (immunoglobulins and antibodies)
ELISPOT	enzyme-linked immunosorbent spot assay (cells producing immunoglobulins or antibodies)
FCS	fetal calf serum
Gli	gliadin
GFD	gluten-free diet
GTF	glycosyltransferase
Gy	Gray
H. influenzae	Haemophilus influenzae
LPS	lipopolysaccharide
ND	normal diet
OD	optical density
Ova	ovalbumin
PNP	paranitrophenylphosphate
PBS	phosphate-buffered saline
PVA	partial villus atrophy
S. mutans	Streptococcus mutans
S. epidermidis	Staphylococcus epidermidis
SVA	subtotal villus atrophy
TBS	tris buffered saline

T_h	T helper cell
T_s	T suppressor cell
Tween	polyoxyethylene sorbitan

Inflammatory Bowel Disease: current status and future approach
R.P. MacDermott, editor

DO SALIVARY ANTIBODIES TO DIETARY ANTIGENS REFLECT INAPPROPRIATE STIMULATION OF GALT OR HYPERPERMEABILITY?

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INTRODUCTION

Since there is traffic of lymphocytes between mucosal surfaces (1) antibodies in saliva may reflect the secretory immune responses of the gastrointestinal tract (2). We are examining the possible role of saliva in this context. In this first phase, a detailed study of salivary immunoglobulins and specific antibodies has been pursued. Pure parotid saliva is a stable, clean, secretory fluid, easily obtained by non-invasive means, requiring litle processing before analysis and in many ways ideal for such investigation.

We selected patients likely to have abnormally permeable gastrointestinal mucosae with, possibly, secretory antibodies to common food proteins. We have also embarked on an investigation of the effects of complete dietary exclusion of the relevant foods on the levels of any antibodies present.

METHODS

Subjects studied were 142 normal volunteers, 14 patients with active coeliac disease, and 12 patients with active small intestinal Crohn's disease. Pure parotid saliva was collected via a Carlsson-Crittenden cup for five minutes, with flow stimulated by two mls. of 5% citric acid in aliquots. The volume was measured by graduated pipette and saliva stored at -20^{o}C.

The protein content was assayed by the Lowry method. Total immunoglobulins IgA, IgG, and IgM were determined by an ELISA technique using a WHO reference immunoglobulin serum as standard. Specific antibodies to gliadin (gli), ovalbumin (ova), and B-lactoglobulin (BLG), were also analysed by ELISA. Although IgA, IgG, and IgM assays were used, we have, to date, limited data for the IgG and IgM food antibodies in the controls population and so only results of assays for IgA specific antibodies are reported.

ELISA results were assessed in relation to volume, flow rate, salivary protein content, and (for the IgA-specific antibodies) in relation to total IgA. Results per ml. are shown. Statistical analysis was by using contingency tables, non-parametric ranking and the Wilcoxon paired rank sum test.

RESULTS

Total immunoglobulins

IgA was present in 137/142 of control and all patient samples, IgM in 97/124 and IgG in 32/155 control samples, in 10/12 and 3/10 of coeliacs, and 12/12 and 4/12 of Crohn's patients, ($p>0.05$, NS).

Concentrations of IgA and IgG were not different, but in contrast to previous reports (3,4), levels of IgM were significantly increased in both patient groups, $p = 0.0018$ and 0.0001 for coeliac and Crohn's disease respectively, (fig. 1). No differences with regard to age, sex, salivary volumes and flow rates, or protein concentration were found.

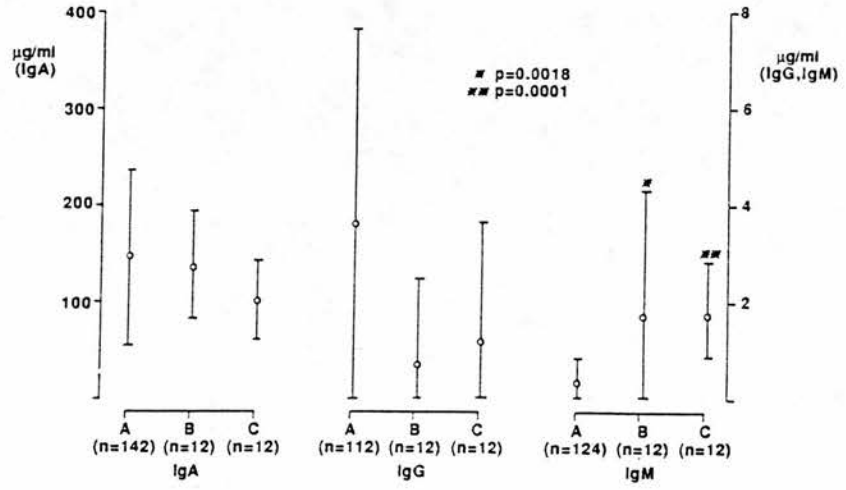

Fig. 1 Total immunoglobulin levels in controls (A), coeliacs (B), and Crohn's disease (C), * = significant difference.

Implications

It is not certain what these markedly raised IgM concentrations represent. Are they a result of disordered immunoregulation, such as a failure of B cell switching, perhaps secondary to defective T_h function? Are they due to the nature of the antigen? Others have found an increase in the number of IgM-producing plasma cells in the jejunal mucosa, and of IgM in jejunal juice of coeliacs (5), and the increased salivary immunoglobulins may reflect this. However the same investigators found no difference in the levels of IgM in the saliva of their subjects.

IgA anti-food antibodies

IgA antibodies to gliadin, ova and BLG were detectable with similar frequency in all three groups. Antibody levels to all three food proteins were significantly increased in Crohn's disease ($p = 0.0018$ gli; 0.005 ova; 0.028 BLG), and to two of the three in coeliac disease ($p = 0.0055$ gli; 0.0007 ova).

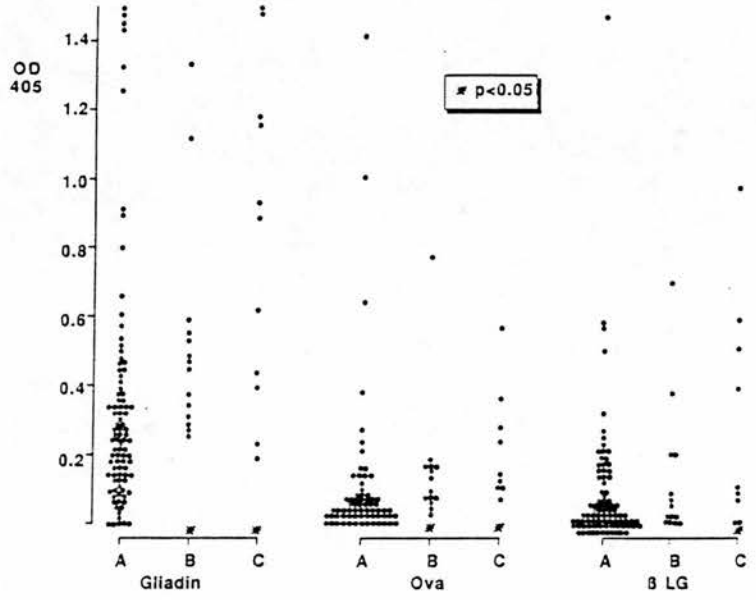

Fig. 2 IgA anti-food antibody levels. (key as fig. 1)
* = significant difference, see text.

Implications

Similar findings in patients with two quite distinct diseases suggests a non-specific mechanism. The most likely explanation is that this a secondary phenomenon following damage to the gut wall, the subsequently increased permeability allowing increased antigenic load to reach the gut lymphoid system. It is unlikely that these antigens have a primary causative role in Crohn's disease, although they may be involved in perpetuation of inflammation after an initial insult. The precise relevance to coeliac disease is unclear, and the presence of detectable antibodies in many normal volunteers, although at lower titres, argues against a pathogenetic role.

IgA anti-gliadin antibodies during elemental diet therapy

In this pilot study of five patients with active small intestinal Crohn's disease, treatment with an elemental diet (Elemental 028) for varying periods was accompanied by a fall in IgA anti-gliadin antibodies, $p = 0.004$, Fig. 3. One patient continued to take small amounts of normal food in addition to the elemental diet; her readings remained unchanged. There was no correlation with total IgA levels.

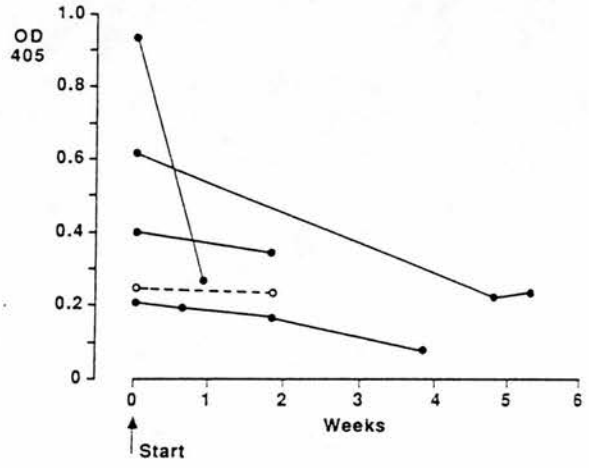

Fig. 3 Change in IgA anti-gliadin antibody levels, elemental diet instituted at day 0. Patient took normal food in addition.

Implications

Withdrawal of gliadin from the diet resulted in a definite though variable decrease in specific antibody levels in saliva. Continued intake of small amounts of gliadin in one patient may have prevented this fall. By such dietary manipulation a means of determining the kinetics of the secretory immune response may be available. Elemental diets have been shown to improve patients with Crohn's disease, who demonstrate relapse after reintroduction of a normal diet. Salivary antibodies to enterically encountered antigens, even if not directly involved in the pathogenesis, may reflect disease activity.

SUMMARY AND CONCLUSIONS

Increased levels of IgM, and IgA anti-food antibodies are found in the parotid saliva of patients with coeliac disease and Crohn's disease. In five patients with active Crohn's disease, IgA anti-gliadin antibody levels fell during a period of treatment with an elemental diet.

Pure parotid saliva can be readily used to measure total immunoglobulins and isotope-specific antibodies to foods. Events in saliva may mirror those in distal gastrointestinal secretions. These preliminary results are encouraging and merit further study; we are now comparing the patterns of antibodies in saliva with those in serum and in gut irrigation fluid.

ACKNOWLEDGEMENTS

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Mucosal immunodeficiency in smokers, and in patients with epithelial head and neck tumours

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Mucosal immunodeficiency in smokers, and in patients with epithelial head and neck tumours

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Abstract

Cigarette smoking influences the risk of orogastrointestinal disease in both protective (ulcerative colitis), and inductive (squamous tumours of the head, neck and oesophagus) roles. In order to study the effects of smoking on mucosal immunity, salivary immunoglobulins were measured in pure parotid saliva from groups of healthy non-smokers, smokers, and exsmokers and from patients with epithelial head and neck tumours, both untreated and after radiotherapy. Of the healthy individuals, smokers had significantly lower salivary IgA and higher IgM concentrations than did non-smokers. The effect on IgA was dose related, and reversible after cessation of smoking. Likewise, in patients with head and neck tumours (the majority being smokers), salivary IgA concentration was reduced and IgM increased when compared with non-smoking controls. Results were similar before and after radiotherapy. This study provides evidence of the effects of smoking on mucosal immunity as evaluated by parotid salivary immunoglobulins. Further studies of the influence of smoking on secretory immunity are indicated.

There is strong epidemiological evidence that smoking has a role in certain inflammatory and malignant orogastrointestinal diseases.[1] The incidence of ulcerative colitis is much lower in smokers than in non-smokers or exsmokers.[2–4] On the other hand smoking is strongly associated with epithelial tumours of the upper orogastrointestinal tract.[1] It is likely to be of relevance to these disease associations that cigarette smoking has profound effects on the immune system, and this has recently been reviewed.[5] Much work has been conducted on systemic immune status but, surprisingly, the influence of smoking on mucosal immunity has been relatively neglected. A recent report that smokers have reduced numbers of antigen presenting cells in the cervical epithelium[6] is of interest.

As part of a programme of research on human mucosal immunity, we developed techniques for the measurements of salivary immunoglobulin concentrations by ELISA. In order to investigate a possible effect of smoking on mucosal immunity we applied these techniques to stored saliva which had been collected in Edinburgh for other purposes from healthy smokers, healthy non-smokers, and patients with epithelial head and neck tumours. Results from these Scottish patients and healthy subjects revealed significant differences between the various groups. To confirm these data and in addition to avoid the possible confounding effects of concurrent alcohol consumption, prospective studies were then performed in Egyptian, non-drinking, healthy subjects and patients with head and neck tumours.

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Methods

EDINBURGH HEALTHY SUBJECTS AND PATIENTS

Healthy smokers and non-smokers were recruited from volunteers, and disease controls from patients attending clinics for obesity, bulimia, and treated hypo and hyperthyroidism. Patients with head and neck tumours were recruited from the Otolaryngology clinics. Patients who had received radiotherapy had been treated with a standard 52·5 Gy course over four weeks in 20 treatments. The field of irradiation did not include the parotid glands. Because of the retrospective nature of this phase of the study alcohol intake was not recorded, smoking history was incomplete for the non-smokers (never smoked or ex-smokers?) and the groups were not age and sex matched. Clinical details are given in Table I.

CAIRO VOLUNTEERS AND PATIENTS

Healthy non-smokers, smokers, and ex-smokers were recruited from clinical, laboratory and ancillary staff, and from people attending for the removal of ear wax at the Ain Shames University Hospitals, Cairo, Egypt. In addition, all patients with epithelial head and neck tumours who were attending the Department of Otolaryngology in

TABLE I *Subject numbers in each group from both centres. ?=status unknown (retrospective data – see text), –=not investigated.*

	Edinburgh			*Cairo*		
Subjects	*M*	*F*	*Total*	*M*	*F*	*Total*
Healthy controls						
Non-smokers	31	83	114	19	11	30
Smokers	19	9	28	27	20	47
Ex-smokers −2 yr	–	–	–	11	10	21
Ex-smokers −5 yr	–	–	–	10	7	17
Age+sex matched for Tumour patients	–	–	–	18	10	28
Tumour patients						
Untreated						
Current smokers	13	4	17	14	4	18
Ex-smokers	?	?	?	7	4	11
Non-smokers	?	?	?	0	1	1
Unknown	5	5	10	0	0	0
Total	18	9	27	21	9	30
Irradiated						
Current smokers	0	0	0	7	2	9
Ex-smokers	?	?	?	14	7	21
Non-smokers	?	?	?	0	0	0
Unknown	26	12	38	0	0	0
Total	26	12	38	21	9	30

these hospitals were invited to participate. These patients received 50–60 Gy in 25–30 treatments over five to six weeks. The field of irradiation included the parotid glands. Healthy subjects and patients did not take alcohol, and non-smoking groups were separately age and sex matched for healthy smokers and for the tumour patients. Of the patients with tumours (untreated and irradiated respectively), 13 and 11 had tumour in the larynx, nine and eight in the hypopharynx, and eight and 11 in the oropharynx.

Of the Cairo volunteers and patients there were 47 smokers mean age 29·8 years, range 21–37 and 30 non–smoking controls, mean age 29·2 (21–36) (age comparison, U=665·5, p=0·682). Mean age of 30 tumour patients was 52·7 (31–64), and of 28 controls, mean 51·6 (34–61), with again no difference between the test groups and their controls in terms of age (U=366, p=0·596). The irradiated group were somewhat older than both the control and the tumour patients, mean age 59·0 (48–73) (U=187·5, p=0·0004 *v* controls; U=250·5, p=0·0033 *v* tumour patients). These patients had tumours in various sites which did not differ between groups (χ^2=0·699, 2 df, p=0·71).

There were no differences in the sex distribution between any group of Egyptian subjects, (χ^2=1·82, 4 df, p=0·7712).

CLINICAL PROCEDURES

Full informed consent was obtained in every case. For each subject details of age, sex, weight, smoking history, general health and, if appropriate, tumour site were recorded. Before collection of saliva the oral cavity was examined for evidence of local sepsis (gingivitis, pharyngitis, tonsillitis, dental abscess: if any of these conditions were present, the person was excluded from the study). Parotid saliva was then obtained using a simple modification of a Carlsson-Crittenden cup (J Harvey, Lochgilphead, Argyll, Scotland). The secretion of parotid saliva was stimulated by 5% citric acid given in four 0·5ml aliquots: collection of saliva was bilateral and simultaneous for two minutes (Cairo) or unilateral for five minutes (Edinburgh group). Sample volumes were measured by graduated pipette, and the samples stored at −70°C until analysis. Flow rates were calculated from the volume in millilitres divided by the collection period in minutes, divided by the number (one or two) of parotid ducts from which saliva was collected.

ASSAY TECHNIQUES

Salivary concentrations of IgA, IgG, and IgM were assayed by a double sandwich ELISA technique. Briefly, microtitre plates M129A (Dynatech, Billingshurst, Sussex) were coated with one in 5000 goat antihuman IgA, IgG, and IgM. The plates were washed five times with 0·9% saline with 0·05% Tween 20 (Sigma, Poole, Dorset), blocked for 20 minutes with the washing solution with 1% adult bovine serum (Scottish antibody production unit, Carluke, Lanarkshire), then pure parotid saliva was applied at a 1:2 dilution for IgG and IgM and a 1:5 dilution for IgA, 125 µl being added to each well, and left for approximately eight hours at 20°C. After further washing five times, alkaline phosphatase conjugated goat antihuman IgA, IgG, and IgM (Northeast Biomedical Laboratories, Uxbridge, Middlesex) was added and incubated overnight at 4°C. Again after washing five times, paranitrophenyl phosphate at a concentration of 1 mg/ml in 10% diethanolamine buffer was added. As a standard control, World Health Organisation human serum immunoglobulin (1st international reference preparation, National Institute for Biological Standards, Potters Bar, Herts) was used. The plates were read at optical density 405 nm on a Dynatech Microelisa Autoreader MR580. Total protein was measured using the Pierce method. Electrolytes were analysed on a standard SMAC II.

STATISTICAL ANALYSIS

The Mann-Whitney U test, Wilcoxon's paired rank-sum test, and Spearman's rank correlation coefficient were used for statistical analysis.

Results

SALIVARY IMMUNOGLOBULIN CONCENTRATIONS

Edinburgh

The results are shown in Table II. Salivary IgA concentrations were decreased in smokers, and in patients with head and neck tumours, both before and after irradiation. Conversely, IgM concentrations were increased in smokers, and in patients with head and neck tumours, with a few extremely high values in the postirradiation group. These results must be interpreted with caution as the age and sex distribution was distinctly different from the healthy controls.

Cairo

Healthy smokers had significantly lower salivary IgA concentrations (Fig 1), and higher salivary IgM (Fig 2), when compared with non-smokers. There was no influence of smoking on salivary IgG concentration, Figure 3. There was a strong inverse correlation between salivary IgA concentration and the number of cigarettes currently smoked daily (10–60), (Fig 4); however, no such relationship existed for salivary IgG, or IgM concentrations. Salivary flow rates were weakly but significantly correlated positively with the number of cigarettes smoked daily (r=0·291, p=

TABLE II *Salivary immunoglobulin concentrations in the Edinburgh subjects. *= significant difference from control values, p<0·0005 in all cases. I=insufficient saliva.*

	Subjects			
	Non-smokers (n=114)	*Smokers (n=28)*	*Tumour patients (n=27)*	*Irradiated patients (n=30)*
IgA	145 (92)	44 (41)*	80 (61)*	87 (49)*
IgM	0·26 (0·4)	1·04 (0·5)*	0·077 (0·2)	3·2 (9·2)
IgG	3·7 (9·5)	0·07 (0·1)	I	I

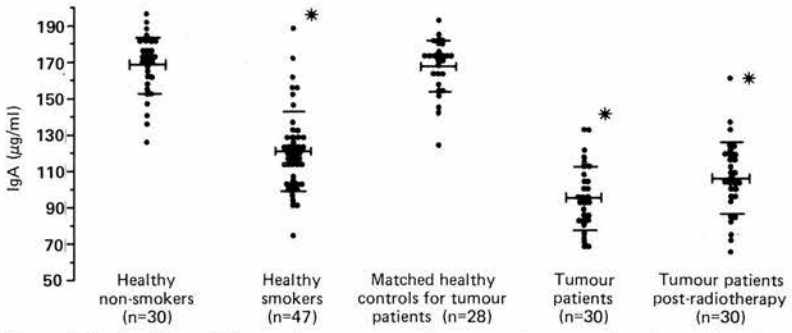

*Figure 1: *=significant difference between appropriate control groups and smokers and patients (U=84, p<0·00001 v healthy smokers; U=2, p<0·00001 v tumour patients; U=9, p<0·00001 v irradiated tumour patients). Bars show mean (standard error).*

0·044) but no relationship existed between salivary immunoglobin concentrations and flow rate.

Salivary IgA concentrations in exsmokers of at least 20 cigarettes daily, who had given up smoking two years before sampling, were higher than in healthy current smokers, and after five years of non-smoking IgA concentrations were similar to those of non-smokers (Fig 5). The effects of stopping smoking on salivary IgM concentrations were less consistent. Values were similar to those for non-smokers after two years' abstinence (U=267, p=0·641, data not shown), but at five years were significantly higher than in non-smokers (U=91, p=0·0004).

There were identical findings when the patients with head and neck tumours (the majority being smokers, or very recent ex-smokers) were compared with the non-smoking controls (Figs 1–3). Again, salivary IgA concentration was markedly decreased and IgM concentration increased, IgG being normal, when compared with values for matched non-smoking controls. No differences were seen between the groups of patients who were untreated, and those who had undergone radiotherapy, Figures 1–3.

Identical results were found in both centres with no differences in salivary protein and electrolyte concentrations between any of the groups.

Discussion

We have shown a striking and reproducible influence of cigarette smoking on salivary immunoglobulins. In healthy smokers, the concentration of IgA in pure parotid saliva is reduced, and of IgM increased, when compared with non-smokers. For IgA, this effect is strongly dose dependent, and reversible; salivary IgA concentrations were similar to those for non-smokers in a group of healthy subjects who had stopping smoking at least five years before. Differences in parotid salivary immunoglobulin concentrations between healthy non-smokers and patients with head and neck cancer might be attributed solely to the smoking habits of the latter. Such a relationship is blurred by the fact that many smokers are heavy alcohol drinkers,[7] and because alcohol consumption (in the absence of hepatic cirrhosis) increases salivary IgA concentrations (Barton and Ferguson, unpublished observations) and is an independant risk factor for squamous cell cancer of the head and neck.[8] We avoided this latter factor by using non-drinkers in the second, case controlled study in which identical results were obtained. Nevertheless in future studies it will be necessary to exclude other environmental factors such as drugs, and genetic factors which influence immunoglobulin levels.

The similarity of the findings between healthy smokers and patients with epithelial head and neck tumours probably reflects the fact that most people who develop these neoplasms smoke. Although many patients in the untreated tumour group and most patients in the postradiotherapy group had stopped smoking at the time of sampling, most commonly at the time of diagnosis, sufficient time may not have elapsed to allow any possible normalisation of salivary immunoglobulin levels.

We are aware of four previous reports of salivary immunoglobulins in smokers,[9–12] but they relate to small numbers of selected patients, and whole saliva was used. Even in the absence of oral sepsis or radiation damage, mixed saliva is contaminated with crevicular fluid, similar in its immunoglobulin content to serum, and is therefore unsuitable as material for the study of mucosal immunity.[13] This is highlighted by the discrepant results in the literature on the influence of smoking on immunoglobulin content of mixed saliva. Bennett found that salivary IgA concentration was low in eight elderly, edentulous male smokers,[11] in contrast with Watanabe who reported high mixed salivary IgA levels in a group of smokers and drinkers, and in head and neck cancer patients.[9] Hersey reported a striking decrease in salivary IgG (probably derived from crevicular fluid or inflammatory exudates) in a small group of healthy smokers who stopped smoking.[10] Olson found no change in whole salivary IgA when smokers stopped smoking and took nicotine or placebo chewing gum.[12] The safe and simple collection of pure parotid saliva provides a clean and stable secretory fluid for assay. This is particularly important in patients with head and neck tumours and after radiotherapy, when there may be immunosuppression and oropharyngeal superinfection.

Studies of serum and circulating leucocytes have been used to determine the effects of smoking on systemic immunity. Smokers have increased polymorphonuclear neutrophil counts, decreased natural killer cell activity, an increase in total T cell numbers with a decrease

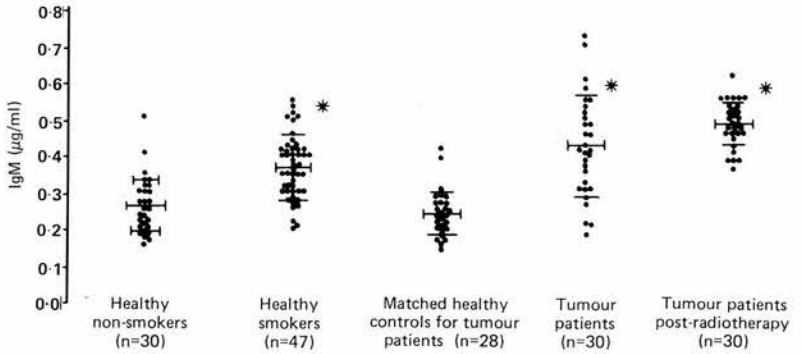

*Figure 2: *=significant difference between appropriate control groups and smokers and patients (U=250, p<0·00001 v healthy smokers; U=113, p<0·00001 v tumour patients; U=11, p<0·00001 v irradiated tumour patients). Bars show mean (standard error).*

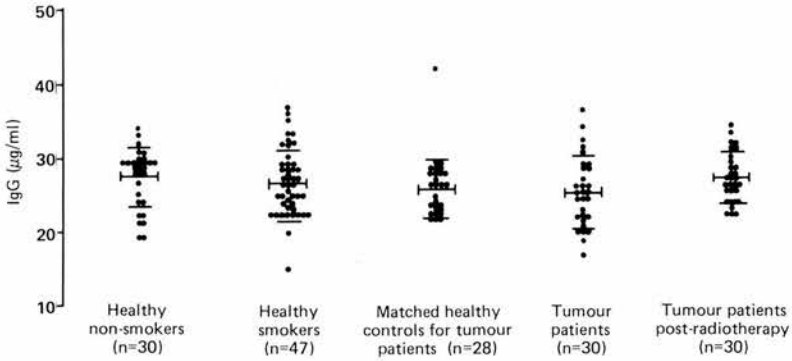

Figure 3: Bars show mean (standard error). No significant differences between groups, ($U=566$, $p=0{\cdot}144$, healthy smokers v controls).

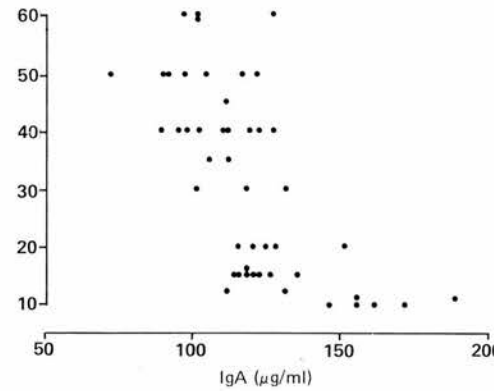

Figure 4: Relationship between daily cigarette consumption and salivary IgA concentration, $r=-0{\cdot}68$, $p<0{\cdot}00001$.

in the T helper/suppressor cell ratio in heavy smokers. In smokers, serum IgA, IgG, and IgM are generally lower by 10–20% than in non-smokers, with higher concentrations of serum IgD and IgE in smokers.[5] Smokers have a generally decreased systemic response to antigens encountered at the nasal and respiratory mucosae,[14,15] although at the mucosal level conflicting results have been reported for total IgA in bronchoalveolar lavage fluid, with a decrease[16] and a three to four-fold increase[17] reported; at the nasal mucosa a decrease in local antibody levels in smokers is reported.[18] In a group of patients with head and neck tumours high whole salivary IgA concentrations were shown to correlate with tumour load, and decrease towards normal after successful treatment.[19]

The increase in IgM concentrations might be interpreted as a compensatory rise. This concept is a little naive; IgM has very different properties to IgA, and its higher concentrations may reflect immunopathology rather than simple compensation. We have not yet addressed the mechanism whereby cigarette smoking alters the immunoglobulin profile of saliva. Intuitively, we favour the concept that smoking impairs T cell immunoregulation of B cell differentiation and maturation, and propose to pursue this hypothesis in the first instance. Alternatively, the alterations may be mediated via the afferent limb of immunity, gut antigen presenting cells as recently reported for uterine cervical epithelium.[6] Both this study and our own suggest a mechanism other than direct toxicity, as the cervix and parotid gland are distant from the local effects of cigarette smoke. It is probably unnecessary to confirm the self reported cigarette consumption; we have no reason to suppose that any errors in reporting would vary between light, moderate or heavy smokers, and others have shown self reported smoking habits to be reliable.[20]

Thus we have shown that smokers have a dose-dependant and probably reversible humoral mucosal immunodeficiency, as reflected either directly or otherwise by salivary IgA concentrations. Studies of mucosal immunity at other surfaces are necessary and should include investigation of T cell factors and other regulatory mechanisms. If changes in secretory immunoglobulins reflect abnormalities of other, as yet unexplored components of mucosal immunity, new avenues of pathogenesis of smoking related diseases may emerge.

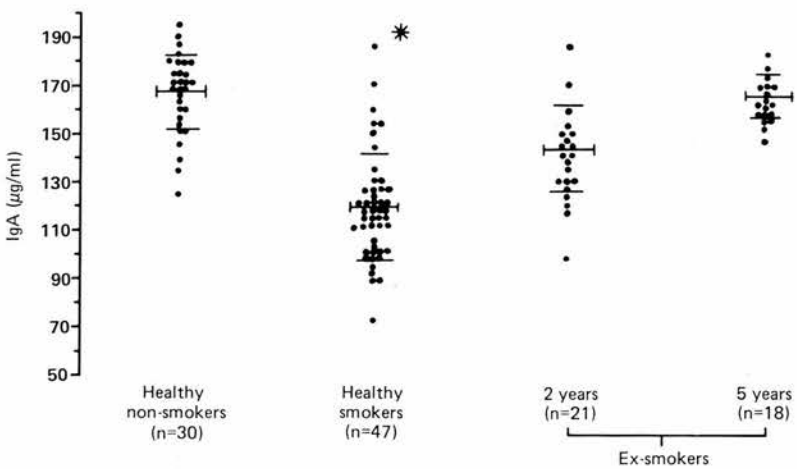

Figure 5: IgA concentrations in ex-smokers of 20 cigarettes daily, after 2 ($U=102{\cdot}5$, $p=0{\cdot}0001$), and 5 ($U=214$, $p=0{\cdot}633$) years abstinence compared with controls. Current smokers are shown for comparison.

We would like to thank Mrs Margaret Gordon and Mrs Jackie Johnston for excellent technical assistance. This work was supported in part by The Scottish Hospitals Endowments Research Trust (SHERT) and by the National Association for Crohn's and Colitis Disease (NACC).

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REGULATION OF ANTIBODIES TO FOOD PROTEINS WITHIN THE COMMON MUCOSAL IMMUNE SYSTEM: LACK OF CORRELATION BETWEEN ANTIBODY TITRES IN SALIVA AND INTESTINAL FLUID

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Previous workers have studied saliva on the assumption that immunological changes reflect events in other gastrointestinal secretions. We tested this hypothesis by comparing total immunoglobulin levels and specific antibody titres in saliva and intestinal fluid obtained by gut lavage from 20 healthy subjects. An ELISA technique was used to measure total immunoglobulins and and specific antibody levels of the IgA, IgG, and IgM isotypes to three common food proteins, gliadin, ovalbumin and beta-lactoglobulin. No correlations between saliva and lavage fluid concentrations of IgA or IgM existed, although a positive correlation was found for IgG which was present in trace amounts in the secretions. Antibody titres in saliva and intestinal fluid did not correlate for any isotype. However in the case of IgM, comparison of saliva with serum, and lavage with serum showed significant positive correlations. This study suggests that saliva does not reflect immune events in intestinal secretions, that the gut must be studied directly, and that regulation of IgM antibody may not be segregated with respect to mucosal and systemic compartments, in contrast to IgA antibodies as is clearly demonstrated.

Introduction

Study of the gastrointestinal mucosal immune system in man has been hindered by practical and ethical problems of access. Investigators have tended to use in vitro studies of isolated tissue specimens obtained from patients at endoscopy or operation which may not be representative. The secretory aspects of gut mucosal immunity have been relatively neglected. The existence of a common mucosal immune system suggests that other mucosal secretions may reflect immunoglobulin and specific antibody status in gut secretions. To determine if parotid saliva is representative of gastrointestinal secretions, we assayed immunoglobulins and antibodies in serum, saliva, and intestinal fluid obtained by whole gut lavage.

Materials and methods

Twenty subjects were recruited for the study, and comprised

healthy volunteers and patients with an eventual diagnosis of functional abdominal problems. The subjects were given the PEG-electrolyte solution (osmolality 260 mosmol/L) and asked to drink at a rate of 1 litre per hour, to a total of 4 litres consumed. The stools were discarded until a clear fluid was passed. Approximately 100-200mls were then collected and immediately treated with soybean trypsin inhibitor, EDTA, phenylmethylsulphonylfluoride, sodium azide and fetal calf serum to inactivate proteases and to prepare a suitable fluid for analysis. This was aliquoted and stored at -70^{0c} until assay. Assay of total IgA, IgG, and IgM, and isotype-specific antibodies of these classes to three food protein antigens, gliadin, ovalbumin, and beta-lactoglobulin was carried out using an ELISA technique. The results for antibody levels were expressed as a percentage of a high positive control.

Results

No correlations existed for antibody titres to the food antigens between saliva and intestinal lavage in any isotype. Antibody titres of the IgM isotype in saliva and intestinal fluid were reflected by serum antibody levels (saliva vs serum antigliadin $r=0.31$, $p=0.011$, $n=20$; antiova $r=0.49$, $p=0.001$, $n=18$; lavage vs serum antiova $r=0.6$, $p=0.013$, $n=9$). Concentrations of total IgG were weakly correlated in saliva and intestinal fluid ($r=0.452$, $p=0.03$), but no such relationship existed for IgA or IgM. There was no correlation in either fluid between total immunoglobulin levels and specific antibody titres.

Discussion

Despite the existence of a common mucosal immune system, this study failed to find any correlation in healthy subjects between two mucosal secretions for either specific antibody titres, or total immunoglobulin concentrations with the exception of IgG. We feel that although antigen- specific effector and memory cells can be found throughout the mucosal immune system after antigen challenge, that final antibody levels may be largely determined by local antigen concentrations. This study suggests that saliva does not reflect immune events in intestinal secretions, that the gut must be studied directly, and that regulation of IgM antibody may not be segregated with respect to mucosal and systemic compartments, in contrast to IgA antibodies as is clearly demonstrated.

Appraisal of gut lavage in the study of intestinal humoral immunity

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ALIMENTARY TRACT

Appraisal of gut lavage in the study of intestinal humoral immunity

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Abstract

Direct investigation of intestinal humoral immunity requires collection of intestinal secretions or mucosal biopsy specimens, or both. A non-invasive technique of gut lavage, with a polyethyleneglycol electrolyte lavage solution as a means of collecting intestinal secretions for immunoglobulin and antibody studies, was evaluated. Fifty patients were studied – 25 immunologically normal patients or volunteers, 15 patients with untreated coeliac disease, and 10 patients with active Crohn's disease. Protease inhibitors were added promptly to samples to prevent proteolysis of immunoglobulin content. Treated lavage samples were assayed by enzyme linked immunosorbent assay for immunoglobulin and antibody content. Studies of serial lavage specimens showed that early, faecally contaminated specimens contained negligible quantities of immunoglobulin, but once the specimens became clear a steady state was reached, with little variation in immunoglobulin content between serial specimens and with a uniform dilution (around 20%) of the ingested polyethyleneglycol. Gut lavage fluid IgA was predominantly secretory, comprising 92%, 81·6%, and 76·7% respectively of the total IgA gut lavage fluid content in the control, coeliac, and Crohn's groups. High values of total IgM and IgA and IgM antigliadin antibodies were detected in the coeliac group, and high values of IgG in the Crohn's disease group. This method of gut lavage is not only an effective bowel cleanser, but also a non-invasive means of obtaining intestinal secretions for the study of humoral immunity in gastrointestinal disease.

The mucosal immune system is largely independent of the systemic immune system. Therefore, for investigation of local immunity in gastrointestinal disease, the gut must be studied directly. Human research in this area has been hampered by difficulty of access to intestinal secretions. Gaspari and colleagues recently described a non-invasive gut lavage and sample processing technique that they performed in 12 healthy volunteers.[1] Human intestinal secretions contain large amounts of proteases,[2 3] and they found that loss of immunoglobulin content due to proteolysis could be prevented by treating specimens with protease inhibitors. We have further evaluated this technique, studying immunoglobulin content and specific antibody (antigliadin) activity in both healthy subjects and patients with gastrointestinal conditions (coeliac disease and Crohn's disease) where aberrant immune regulation is thought to play a pathogenic role.

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Subjects and methods

SUBJECTS

Gut lavage was performed in 50 subjects. Twenty five were immunologically normal patients (16 women, nine men, median age 52, range 21–92 years) who underwent lavage as a bowel preparation for barium enema or colonoscopy, or who volunteered when invited. (No organic pathology was found in these patients, and final diagnosis in most was functional bowel disorder.) Fifteen subjects were patients with untreated coeliac disease (nine women, six men, median age 43, range 28–62 years), all of whom had had a recent jejunal biopsy specimen showing subtotal or severe partial villous atrophy. Ten patients with active Crohn's disease were also studied (four women, six men, median age 27, range 18–92 years).

LAVAGE SOLUTION

The lavage solution is a polyethyleneglycol electrolyte lavage solution. This is available commercially in the USA (Golytely) and is widely used as a method of cleansing the colon for barium enema and colonoscopy.[4] The lavage solution was formulated by the hospital pharmacy and contained 59·1 g polyethyleneglycol 3350 (a mixture of different sized molecules with a mean molecular weight between 3200 and 3700),[5] potassium chloride BP 0·75 g, sodium chloride BP 1·45 g, sodium bicarbonate BP 1·63 g, and sodium bisulphate BP 5·68 g, made up to 1 litre with a resultant osmolality of 260 mOsm/l.

LAVAGE PROTOCOL

After an overnight fast, lavage was begun at 8 30 am. Patients were asked to drink the lavage solution at a rate of 250 ml every 15 minutes for a period of four hours, making the total volume consumed 4 litres. The solution was found to be more palatable if consumed chilled and flavoured with a little lemon juice. In patients in whom

lavage was used as a bowel preparation, one sachet of sodium picosulphate (Picolax) was given later in the afternoon of the same day.

SPECIMEN PROCESSING

Stool collection was begun once the material passed per rectum became liquid, clear, and free of faecal material. Approximately 200 ml were collected and filtered into 50 ml polypropylene tubes. Specimens were centrifuged twice and treated with protease inhibitors as described by Gaspari *et al.*[1] Treated samples were aliquoted and stored at −70°C. In four subjects, early faecal specimens were collected and processed along with later clear specimens. In 10 subjects, the first clear specimen was divided into three aliquots – one was treated immediately, but treatment was delayed for one and two hours each in the other two to determine the time course of sample deterioration due to proteolysis. In 14 subjects, two serial clear specimens were collected to determine whether immunoglobulin content varied between specimens.

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Total IgA, IgM, and IgG; secretory IgA. Assays were performed in 96 well microtitre ELISA plates (Dynatech). All reactants were added in volumes of 0·125 ml per well and all washes were done three times using saline with 0·05% Tween 80 added. For the assay of total IgA, wells were coated with 100 ng/ml affinity purified goat antihuman IgA (Northeast Labs) in 0·1 M carbonate buffer, pH 9·6, and were incubated overnight at 4°C and washed. After washing, serial twofold dilutions ranging from 1250–19·35 ng/ml of a human colostral IgA standard (Sigma) and serial dilutions of treated lavage samples (initial dilution 1/100) were added to the coated wells. Plates were incubated overnight at 4°C and washed. Goat antihuman IgA conjugated with alkaline phosphatase (Northeast Labs) diluted (in saline with 1% fetal calf serum and 0·05% Tween 80) to a predetermined optimal level was added and plates were incubated for three hours at 20°C. After washing, paranitrophenylphosphate (PNPP, Sigma) 1 mg/ml in 10% diethanolamine (DEA) buffer, pH 9·8, was added. Plates were read at an optical density of 405 in an MR580 microELISA reader (Dynatech). A standard curve was constructed; the IgA content of any given sample was determined by taking the mean IgA content of those two sample dilutions whose optical density fell within the range of the standard curve. The method used to determine total IgM and IgG was similar; human reference serum (Protein Reference Unit, Sheffield) was used as a standard, and the initial sample dilution was 1/25.

The assay for secretory IgA[6] was similar to that for total IgA; wells were coated with a goat antihuman secretory piece (Atlantic Antibodies), the same human colostral IgA was used as a standard.

Antigliadin antibodies. The assay was similar to that described above. Wells were coated with crude gliadin at a concentration of 5 μg/ml. Serum from a patient with untreated coeliac disease was used as a positive standard, and lavage samples were added in duplicate dilutions of 1/2 (this dilution gave optimal optical density readings). Plates were read when the standard reached an arbitrary optical density of 1·0; antibody levels are given in the form of optical density readings expressed as percentages of the optical density of the standard.

MEASUREMENT OF POLYETHYLENEGLYCOL VALUES IN LAVAGE SAMPLES

Polyethyleneglycol was quantified by the method of Malawer and Powell,[7] a modification of the turbidimetric analysis of Hyden.[8]

STATISTICAL ANALYSIS

Differences in IgA content of serial lavage specimens were analysed with the paired *t* test. Differences in immunoglobulin and antibody values between patient groups were analysed with the Mann-Whitney U test.

Results

POLYETHYLENEGLYCOL CONCENTRATIONS IN TREATED SAMPLES

Mean (SD) polyethyleneglycol concentration in the processed samples of 25 subjects was 37·3 (3·4) g/l. The initial concentration of 59·1 g/l in the ingested lavage fluid would be reduced to 47·5 g/l by the addition of protease inhibitors, etc; thus, the liquid stools were comprised on average of 78·5% polyethyleneglycol lavage solution and 21·5% gastrointestinal secretions. There was little variation about the mean (Fig 1).

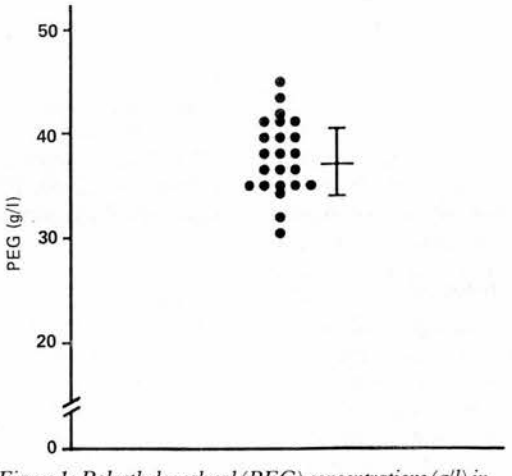

Figure 1: Polyethyleneglycol (PEG) concentrations (g/l) in treated lavage samples (n=25).

IMMUNOGLOBULIN CONTENT IN SERIAL SPECIMENS

Early faecally contaminated specimens contained low concentrations of IgA compared with later clear specimens (Table I). Once the specimens became clear, there was no significant difference in IgA content between serial specimens (Fig 2).

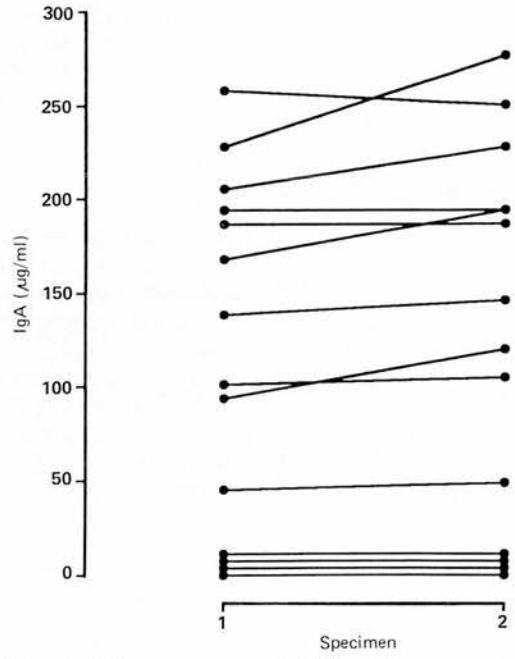

Figure 2: IgA concentrations (μg/ml) in serial lavage specimens (n=14).

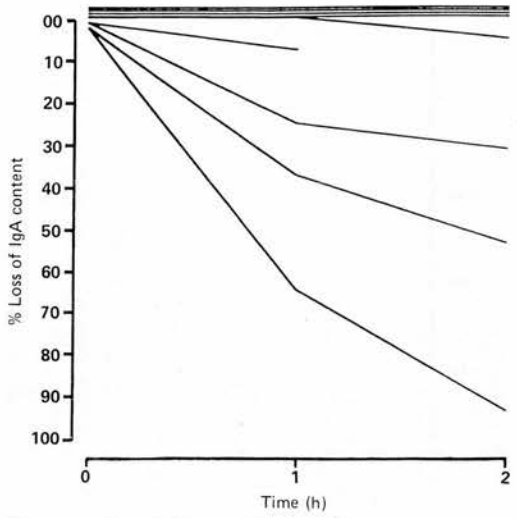

Figure 3: Effect of delay in addition of protease inhibitors on lavage fluid IgA content.

PROTEASE INHIBITION

Delays of one and two hours in the addition of protease inhibitors resulted in loss of IgA content varying from 0 to 92% (Fig 3).

IMMUNOGLOBULIN AND ANTIBODY VALUES IN THE PATIENT GROUPS

Mean immunoglobulin values are shown in Table II. No significant difference in IgA values was found between the three groups. High IgM values were found in the coeliac group ($p<0{\cdot}02$ *v* control subjects) and in the Crohn's patients ($p<0{\cdot}05$ *v* control subjects), with no significant difference between the coeliac and Crohn's groups. High IgG values were found in the Crohn's patients ($p<0{\cdot}00001$ *v* control subjects; $p<0{\cdot}0005$ *v* coeliac group).

Antigliadin antibody values are shown in Figure 4. High IgA antigliadin antibody values were found in the coeliac patients ($p<0{\cdot}002$ *v* control subjects, $p<0{\cdot}01$ *v* Crohn's patients), with similar values in control subjects and Crohn's patients. High IgM antigliadin antibody values were also found in the coeliac patients ($p<0{\cdot}0002$ *v* control subjects, $p<0{\cdot}02$ *v* Crohn's patients), with similar values in control subjects and Crohn's patients.

SECRETORY IgA CONTENT

The mean (range) percentage of lavage fluid total IgA expressing secretory component was as follows: control subjects: 92·0% (82·3–100·0); coeliac patients: 81·6% (57·8–98·7); and Crohn's patients: 76·7% (64·0–91·3).

TABLE I *IgA concentration (μg/ml) in early faecal specimens compared with clear lavage specimens*

Patient	*Early specimen*	*Clear specimen*
1	3·3	16·3
2	<2·0	83·6
3	3·8	18·1
4	<2·0	91·4

TABLE II *Lavage fluid immunoglobulin concentrations (mean (SE)) in μg/ml*

Group	*IgA*	*IgM*	*IgG*
Control (n=25)	136·9 (20·6)	9·26 (2·02)	2·38 (0·72)
Coeliac (n=15)	173·6 (33·8)	23·66 (6·69)*	5·57 (2·42)
Crohn's (n=10)	176·1 (58·0)	17·82 (4·5)*	28·93 (6·75)*

*Values significantly higher ($p<0{\cdot}05$) compared with controls.

Discussion

Investigation of intestinal humoral immunity presents major logistic problems. Some investigators have extrapolated information from studies of peripheral blood and saliva as representative of immune events in the gut. Although this approach may be valid in certain instances – for example, in the study of IgA secreting B cells after enteral infection or immunisation[9] – for definitive studies of intestinal immunity, the gut must be studied directly. For example, we have found no correlation between systemic and mucosal (salivary and jejunal aspirate) antibodies in coeliac disease (unpublished observations).

The gut lavage method described is safe, non-invasive, and useful for bowel cleansing and treatment of constipation.[10] The technique is well tolerated by most patients. We have now carried out gut lavage in over 100 subjects, and the procedure was abandoned in only two patients, who were unable to consume the volume of liquid required. Polyethyleneglycol electrolyte lavage solutions are not absorbed and have minimal effects on fluid and electrolyte balance.[11,12] Other investigators have used saline lavage for collection of intestinal secretions,[13] but this is contraindicated in patients with cardiac, renal, and hepatic disease.

Processing of specimens is crucial to success in subsequent laboratory assays. Several studies have shown that human intestinal secretions contain large amounts of protease,[14] and that the addition of protease inhibitors prevents appreciable immunoglobulin loss[1] and improves antigen stability for ELISA.[3] We and others[1] have shown

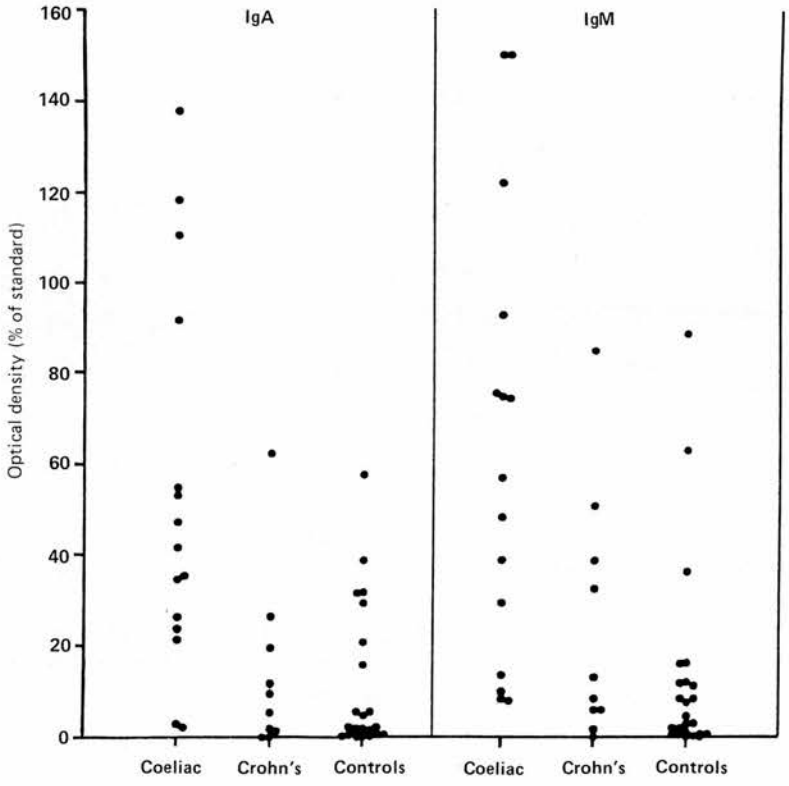

Figure 4: Lavage fluid IgA and IgM antigliadin antibody values.

that delay in addition of protease inhibitors to secretions obtained by this method may result in significant loss of immunoglobulin content.

Results of total IgA content of serial specimens indicates that once the patient passes clear fluid per rectum, a steady state is reached, with no difference in immunoglobulin content between subsequent specimens. Analysis of semiliquid early faecal specimens, however, showed negligible quantities of IgA compared with that found in clear specimens. This must cast doubt on attempts to assess mucosal immunity by studies on faeces.

These preliminary studies on patients with coeliac disease and Crohn's disease have shown substantial qualitative differences between patient groups in patterns of isotype specific immunoglobulins, and these findings are being confirmed in continuing definitive investigations. Specific antibodies can also be readily measured, as we have shown with gliadin and others have shown with cholera toxin B subunit.[1]

It could be argued that increased intestinal permeability in patients with coeliac disease and Crohn's disease may allow serum leakage of immunoglobulin and antibody into the gut lumen. It is quite possible that at least some lavage fluid immunoglobulin is serum derived, but we have shown that lavage fluid IgA is predominantly secretory in the three patient groups.

The finding of high values of lavage fluid IgG in the Crohn's group could reflect altered intestinal immunoglobulin production in this condition;[15] however, as there is no specific mucosal transport system for IgG, it is more likely that the IgG is serum derived, leaking across the inflamed mucosa.

This technique has great potential for in vivo determination of immunoglobulins and antibody in the gut, and can be applied to a wide range of studies of secretory immunity in health and disease.

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Dissociation between systemic and mucosal humoral immune responses in coeliac disease

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Dissociation between systemic and mucosal humoral immune responses in coeliac disease

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Abstract

We examined humoral immunity in coeliac disease as expressed in serum (systemic immunity), and in saliva, jejunal aspirate, and whole gut lavage fluid (mucosal immunity). The aims were to define features of the secretory immune response (IgA and IgM concentrations and antibody values to gliadin and other food proteins measured by enzyme linked immunosorbent assay (ELISA)) in active disease and remission, and to establish whether secretions obtained by relatively non-invasive techniques (saliva and gut lavage fluid) can be used for indirect measurements of events in the jejunum. Serum, saliva, and jejunal aspirate from 26 adults with untreated coeliac disease, 22 treated patients, and 28 immunologically normal control subjects were studied, together with intestinal secretions obtained by gut lavage from 15 untreated and 19 treated patients with coeliac disease and 25 control subjects. Jejunal aspirate IgA and IgM and gut lavage fluid IgM concentrations were significantly raised in patients with untreated coeliac disease; the lavage fluid IgM concentration remained higher in patients with treated coeliac disease than in controls. Serum and salivary immunoglobulin concentrations were similar in the three groups. Patients with untreated coeliac disease had higher values of antibodies to gliadin compared with treated patients and control subjects in all body fluids tested; these were predominantly of IgA and IgG classes in serum, and of IgA and IgM classes in jejunal aspirate and gut lavage fluid. Values of salivary IgA antibodies to gliadin were significantly higher in untreated coeliacs, though antibody values were generally low, with a large overlap between coeliac disease patients and control subjects. In treated patients, with proved histological recovery on gluten free diet, serum IgA antigliadin antibody values fell to control values, though serum IgG antigliadin antibody values remained moderately raised. In contrast, there was persistence of secretory antigliadin antibodies in treated patients (particularly IgM antibody) in both jejunal aspirate and gut lavage fluid. Antibody responses to betalactoglobulin and ovalbumin were similar to those for gliadin, including persistence of high intestinal antibody values in patients with treated coeliac disease. There was a positive correlation between antibody values in jejunal aspirate and gut lavage fluid, but not between saliva and jejunal aspirate; thus salivary antibodies do not reflect intestinal humoral immunity.

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Systemic humoral immunity in coeliac disease has been the subject of intensive investigation. Numerous studies have established that patients with untreated coeliac disease have high values of circulating antibodies to wheat derived proteins such as gliadin, and that antibody values fall after a period of treatment with a gluten free diet.[1–4] Estimation of serum IgA antigliadin antibody is now routinely used both as a screening test for coeliac disease and as a means of assessing dietary compliance.

In contrast, information on mucosal immunity in coeliac disease is patchy. There have been many studies of mucosal lymphoid cells[5 6] and there is circumstantial evidence of a local cell mediated immune response to gluten.[7 8] Several studies have carefully mapped the numbers of Ig producing plasma cells in the jejunal mucosa of patients with untreated and treated coeliac disease,[9 10] showing that untreated patients have increased numbers of IgA and IgM (and to a lesser extent, IgG) jejunal plasma cells. In the 1970s, the presence of intestinal antibodies to food antigens was recognised by a relatively insensitive precipitin technique,[11] but there are only two studies published,[12 13] both in children, on the isotype of antibodies to dietary antigens in intestinal secretions, and these give conflicting results.

The general objectives of this study were twofold. In relation to coeliac disease, our aim was to characterise, in vivo, intestinal humoral immunity. Total immunoglobulins and specific antibodies to gliadin and to two antigens which are not toxic in coeliacs were measured in three different mucosal secretions. Untreated and treated patients with coeliac disease were studied to determine whether abnormalities of secretory immunity are permanent and intrinsic to the coeliac diathesis or are only present in active disease. Separately, and of relevance to the clinical investigation of mucosal immunity, we studied the relations between systemic and intestinal antibodies, and we examined our data to establish whether patterns of immunoglobulins and antibodies in jejunal fluid are mirrored in other secretions which can be obtained without intubation. The salivary glands are considered part of the common mucosal immune system,[14] and we therefore studied pure parotid saliva. We also used a whole gut lavage technique for the non-invasive collection of intestinal secretions.[15]

Methods

PATIENTS STUDIED AT THE TIME OF JEJUNAL BIOPSY

Specimens of saliva, jejunal aspirate, and serum were collected at the same time as jejunal biopsy on 76 occasions in 69 patients. There were 41 patients with coeliac disease (seven studied twice), (23 women and 18 men; median age 42 years, range 15–78) and 28 control patients (14

women, 14 men, median age 35 years, range 14–75). Control subjects had jejunal biopsy to exclude coeliac disease – jejunal histology in these patients was normal, no other significant pathology was found, and a final diagnosis of functional bowel disease was made. Twenty six of the patients with coeliac disease were untreated and histological examination of the jejunal biopsy specimens showed subtotal or severe partial villous atrophy. Seven of these and a further 15 patients with treated coeliac disease (all with previous diagnostic biopsy specimens) underwent biopsy again while on a gluten free diet. The median period on gluten free diet was three years (range 3 months – 17 years). Eleven had entirely normal jejunal histology (all of these had been taking a gluten free diet for at least two years), and 11 had minor histological changes – for example increased intraepithelial lymphocytes (most patients in this group had been taking a gluten free diet for less than one year).

PATIENTS STUDIED BY WHOLE GUT LAVAGE

Gut lavage was carried out in 15 untreated coeliac disease patients, 19 with treated disease, and 25 control patients. These included 10, eight, and two patients respectively from each group who had also had collection of jejunal aspirate. The median period on a gluten free diet in patients with treated coeliac disease undergoing gut lavage was eight years (range 3 months – 19 years). Eleven of the patients on a gluten free diet had in the past shown a clinical and histological response to the diet, but did not undergo biopsy again at the time of this study. Gut lavage was carried out in 25 control patients (16 women and nine men, median age 52, range 21 – 92 years). These subjects were either healthy volunteers or patients with functional bowel disorder.

SPECIMEN COLLECTION AND PROCESSING

Saliva: parotid salivary flow was stimulated with 5% citric acid sublingually in four 0·5 ml aliquots over five minutes, and collected via a Carlsson-Crittenden cup placed over the parotid duct, with gentle aspiration to maintain position and suction. We collected stimulated saliva only.

Jejunal aspirate: samples were collected from a point just distal to the duodenal-jejunal junction, through the tubing of the Crosby capsule, before taking the biopsy specimen. The protease inhibitor phenylmethyl sulphonylfluoride (PMSF, Sigma) 100 mM in 95% alcohol (20 µl per ml of aspirate) was added before aliquoting.[16] Serum was obtained from all patients.

Gut lavage: the lavage fluid used was a polyethylene glycol (PEG) based electrolyte lavage solution (Golytely). After an overnight fast, patients drank this solution at a rate of 250 ml every 15 minutes for a period of four hours, making the total volume consumed four litres. Specimen collection began once the material passed per rectum became liquid, clear, and free of faecal material. Approximately 200 ml was collected and filtered into 50 ml polypropylene tubes; specimens were centrifuged and treated with protease inhibitors as described by Gaspari *et al.*[15]

All the above specimens were aliquoted and stored at −70°C.

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Reference materials and reporting of results

For assays of immunoglobulins in the various secretions, serial twofold dilutions of a standard preparation were used to produce a standard curve. For example, for IgA assays dilutions ranging from 1250–19·35 ng/ml of a human colostral IgA standard (Sigma) were used in each test run. Serial dilutions of test samples (varying in initial dilution depending on the type of specimen) were also assayed. Only when the optical density results of at least two of these sample dilutions fell within the range of the standard curve was the assay considered technically satisfactory. The IgA content of the sample was then determined by taking the mean IgA content of these two sample dilutions. For total IgM and IgG in secretions human reference serum (Protein Reference Unit, Sheffield) was used as a standard.

In the assays of specific antibodies, experiments were carried out to define optimal test conditions for each antigen, isotype, and secretion. Serum from a patient with untreated coeliac disease, previously recognised as having high titres of antibodies of all isotypes to a wide variety of dietary antigens, was used as a reference standard. The reference specimen and test specimens were studied at suitable dilutions, varying for the different assays, and the plates were read when the optical density for the standard reached 1·0. Results for test specimens are expressed as optical density readings, % of this standard. Results are thus expressed as non-parametric data; antibody values are not directly proportional to the antigen binding capacity of the sample. This is a feature of all such assays.

Immunoglobulins (jejunal aspirate, gut lavage fluid, and saliva)

Assays were performed in 96 well microtitre ELISA plates (Dynatech). All reactants were added in volumes of 0·125 ml per well and all washes were done three times using saline with 0·05% Tween-80 added. For the assay of total IgA, wells were coated with 100 ng/ml affinity purified goat antihuman IgA (Northeast Labs) in 0·1 M carbonate buffer, pH 9·6, and incubated overnight at 4°C and washed. After washing, serial twofold dilutions of standard and samples (initial sample dilution 1/100) were added to the coated wells. Plates were incubated overnight at 4°C and washed. Goat antihuman IgA conjugated with alkaline phosphatase (Northeast Labs) diluted (in saline with 1% fetal calf serum and 0·05% Tween-80) to a predetermined optimal value was added and plates were incubated for three hours at 20°C. After washing, paranitrophenylphosphate (PNPP, Sigma) 1 mg/ml in 10% diethanolamine (DEA) buffer, pH 9·8, was added. Plates were read at optical density 405 in an MR580 microELISA reader (Dynatech). The IgA content of any given sample was determined as described above.

The method used to determine total IgM and IgG in secretions was similar; initial sample dilution was 1/25.

Serum immunoglobulins were measured by autoanalyser using an immunoturbidimetric method.

Food antibodies

The assay was similar to that described above. Immulon 2 (129B) ELISA plates (Dynatech) were used. Wells were coated with antigen (gliadin, betalactoglobulin and ovalbumin) at a concentration of 5 μg/ml. Betalactoglobulin and ovalbumin were supplied by Sigma; gliadin was supplied by Dr Stefan Strobel. Reference standard and samples were added in duplicate dilutions to the coated wells. The following sample dilutions were used: serum: 1/100 (IgA and IgM) and 1/200 (IgG); jejunal aspirate: 1/10; gut lavage fluid: 1/2; saliva: 1/2. An appropriate dilution of standard was included in each assay; these dilutions gave optimal optical density readings.

We established that these antibodies were specific by incubating samples with the relevant antigen and showing that the antibody was specifically absorbed out. In a study of 20 samples, the within plate optical density coefficient of variation was 7·3%, and the between plate optical density variation was 11·1%. If the optical densities of the duplicate sample dilutions varied by >15%, the assay was repeated.

TABLE I *Immunoglobulin concentrations (median (range)) in serum (mg/ml), jejunal aspirate, gut lavage fluid, and saliva (μg/ml)*

	Ig class	*Untreated coeliac patients*	*Treated coeliac patients*	*Control subjects*
Serum	IgA	2·4 (1·3–3·8)	2·0 (0·7–3·3)	1·8 (0·9–4·1)
	IgM	1·1 (0·3–3·4)	0·9 (0·3–3·1)	1·3 (0·3–4·5)
	IgG	10·5 (5·6–15·7)	10·7 (5·4–20·1)	9·5 (7·3–15·9)
Jejunal aspirate	IgA	290* (25–2282)	140·7 (20–1558)	102·5 (22·9–540·6)
	IgM	29·8*† (1·9–357·9)	9·6 (0–174·4)	4·5 (0–39·5)
	IgG	23·9* (3–261·8)	11·8 (0·3–271·4)	7·3 (1·2–29·3)
Gut lavage fluid	IgA	146·2 (10·7–487·5)	90·8 (8·8–621·8)	139·7 (7·9–403)
	IgM	17·5* (2·1–100·1)	25·7* (0–192·2)	5·3 (0–37)
	IgG	2·7 (0·1–34·9)	0·9 (0·1–11·4)	1·0 (0–12·2)
Saliva	IgA	123·8 (48·6–454)	118·1 (32·2–838·2)	165·8 (66·5–606·9)
	IgM	1·2 (0·1–138·9)	1·4 (0·1–12·4)	1·1 (0·1–3·6)
	IgG	1·3 (0–25·1)	0·9 (0·1–10·6)	0·5 (0–34·3)

*Immunoglobulin concentration significantly higher ($p<0·05$) than in control subjects.
†Immunoglobulin concentration significantly higher ($p<0·05$) than in treated coeliac disease patients.

TABLE II *Serum food antibody values (median (range))*

Antigen	*Ig class*	*Untreated coeliac patients (n=26)*	*Treated coeliac patients (n=22)*	*Control subjects (n=28)*
Gliadin	IgA	43·7*† (4·6–150)	5·8 (0·3–41·9)	5·3 (0–44·5)
	IgM	43·0 (19·3–95·4)	38·6 (10·5–87·6)	47·9 (16·1–108·8)
	IgG	82·4*† (9·8–137·4)	41·6* (6·8–107·4)	21 (0–105·2)
BLG	IgA	14·7*† (1·4–150)	6·6* (2·1–87·5)	3·3 (0·2–36·4)
	IgM	33·6 (9–125·5)	38·7 (11·3–94·6)	27·4 (3·5–82·2)
	IgG	94·7* (5–150)	70·2 (8·6–150)	30·9 (0–131·8)
OVA	IgA	18·8* (2·6–150)	14·8 (4·8–60·6)	11·4 (0·9–35·9)
	IgM	31·1 (4–87)	37·8 (9·5–123·4)	30·0 (5·7–102·6)
	IgG	64·4 (2·6–150)	63·4* (8·4–150)	27·6 (3·6–150)

*Antibody values significantly higher ($p<0·05$) than in control subjects.
†Antibody values significantly higher ($p<0·05$) than in treated coeliac disease patients.
BLG=betalactoglobulin; OVA=ovalbumin.

TABLE III *Jejunal aspirate antibody*

Antigen	*Ig class*	*Untreated coeliac patients (n=26)*	*Treated coeliac patients (n=22)*	*Control subjects (n=28)*
Gliadin	IgA	26·5*† (0·7–134·5)	4·0* (0·3–34·2)	1·1 (0–13·3)
	IgM	80·5*† (0·1–150)	19·6* (2·1–114·4)	1·9 (0–11·7)
	IgG	1·8*† (0–23·4)	1·0* (0–4·8)	0·2 (0–2·2)
BLG	IgA	25·3* (0·6–150)	9·2 (1·1–110·9)	5·0 (0·1–60·8)
	IgM	16·4* (0–150)	9·2* (1·1–78·3)	1·6 (0–11·7)
	IgG	4·5* (0–104)	1·6 (0–35·1)	1·0 (0–7·7)
OVA	IgA	15·8* (4–150)	19·6* (1·5–66·5)	4·2 (0–100·5)
	IgM	18·7* (0–97)	8·4* (0·1–52.5)	1·1 (0–17·7)
	IgG	1·3* (0–26·2)	1·1 (0–11·7)	0·5 (0–26·5)

*Antibody values significantly higher ($p<0·05$) than in control subjects.
†Antibody values significantly higher ($p<0·05$) than in treated coeliac disease patients.
BLG=betalactoglobulin; OVA=ovalbumin.

STATISTICAL METHODS

Differences in antibody values and immunoglobulin content were analysed using the Mann-Whitney U test. For correlations, Spearman's test was used.

Results

IMMUNOGLOBULIN CONCENTRATIONS (TABLE I)

Serum

No significant differences were observed.

Jejunal aspirate

Untreated coeliac disease patients had significantly higher jejunal aspirate concentrations of IgA, IgM, and IgG compared with controls, and higher IgM compared with patients with treated coeliac disease. Values for treated coeliac disease patients were not significantly different from those of control subjects.

Gut lavage fluid

IgM content was significantly higher in both untreated and treated coeliac disease patients than in control subjects .

Saliva

No significant differences were observed.

ANTIBODIES TO FOOD PROTEINS

Serum (Table II)

Untreated coeliac disease patients had high values of serum IgA antigliadin antibody, with values for treated coeliac disease patients similar to control values. High values of serum IgG antigliadin antibody were found in both untreated and treated coeliac disease patients, with significantly higher values in the untreated patients. There were no significant differences between patient groups in values of serum IgM antibodies. Patterns of serum antibodies to OVA and betalactoglobulin were generally similar to those for antigliadin antibody, as detailed, with statistical information in Table II. Untreated patients had high values for IgA and IgG anti-betalactoglobulin antibody and IgA anti-ovalbumin antibody. Serum IgA anti-betalactoglobulin and IgG antiovalbumin antibody values were higher in treated patients than in control subjects.

Jejunal aspirate (Table III)

There was very little antibody detected in jejunal

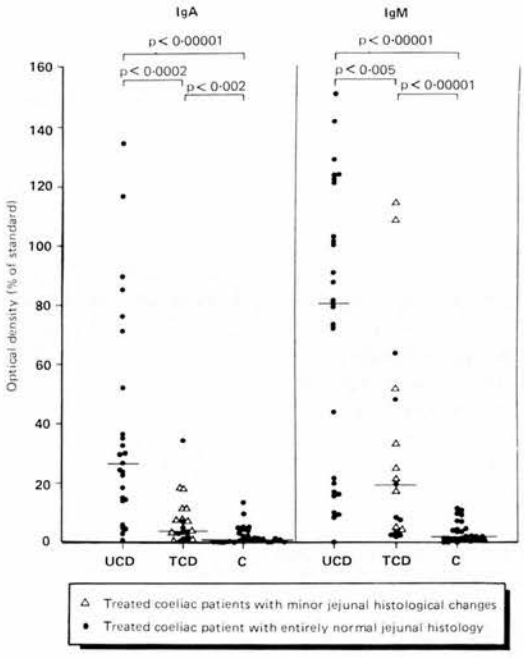

Figure 1: Jejunal aspirate IgA and IgM antigliadin antibody values. (Median antibody values shown as horizontal bars.)

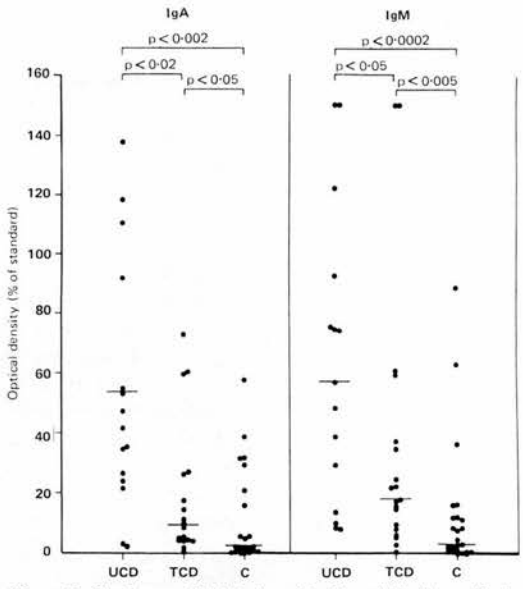

Figure 2: Gut lavage fluid IgA and IgM antigliadin antibody values. (Median antibody levels shown as horizontal bars.)

aspirates from control subjects, but as detailed in Table III, for all three isotypes and all three antigens studied, antibody values were significantly higher in jejunal aspirates from untreated patients than from control subjects (p values all <0·02). For antigliadin antibodies, values in treated coeliac patients were intermediate between untreated coeliac disease patients and control subjects and significantly different from both. When antibody values in the 11 treated patients with entirely normal jejunal histology were compared with those in control subjects, IgA antigliadin antibody values were not significantly higher. Conversely, IgM antigliadin antibody values remained significantly raised (p<0·005) in this group. Antibodies to betalactoglobulin and ovalbumin showed a greater overlap between values in coeliac disease patients and control subjects, but again high IgM antibody values persisted in the treated patients.

Jejunal aspirate IgA and IgM antigliadin antibody values are shown in Figure 1.

Gut lavage fluid (Table IV)

As in jejunal aspirate, high values of IgA and IgM antibodies to gliadin were found in both untreated and treated coeliac disease patients compared with control subjects, with significantly higher antibody values in the untreated compared with the treated patients. High values of IgA and IgM antibodies to betalactoglobulin and ovalbumin were found in untreated patients; high values of IgA and IgM anti-ovalbumin and IgM anti-betalactoglobulin antibodies persisted in the treated coeliac disease patients.

Gut lavage fluid IgA and IgM antigliadin antibody values are shown in Figure 2.

TABLE IV *Gut lavage fluid antibody values*

Antigen	*Ig class*	*Untreated coeliac patients (n=15)*	*Treated coeliac patients (n=19)*	*Control subjects (n=25)*
Gliadin	IgA	53·6*† (2·2–137·8)	9·2* (0·4–72·9)	2·2 (0–57·9)
	IgM	56·9*† (8–150)	17·9* (0·2–150)	3·1 (0–88·7)
	IgG	0·4* (0·2–19)	0·6 (0–3)	0·2 (0–6·5)
BLG	IgA	7·9* (1·8–85·5)	8·4 (0·6–76·5)	3·5 (0·1–71·2)
	IgM	5·6* (0–14·5)	5·3* (0–53)	0·1 (0–22·6)
	IgG	0·7 (0–21)	0·4 (0–8·1)	0·4 (0–4·4)
OVA	IgA	15·0* (3·7–39·3)	14·7* (0·5–46·8)	4·8 (0–76)
	IgM	6·6* (0·8–33·3)	3·6* (0–46·3)	0·9 (0–20·6)
	IgG	0·3* (0–1·3)	0·0 (0–61·2)	0·0 (0–3·6)

*Antibody values significantly higher (p<0·05) than in control subjects.
†Antibody values significantly higher (p<0·05) than in treated coeliac disease patients.

TABLE V *Salivary antibody values*

Antigen	*Ig class*	*Untreated coeliac patients (n=26)*	*Treated coeliac patients (n=22)*	*Control subjects (n=28)*
Gliadin	IgA	5·1*† (0·1–29·6)	4·2 (0–12·5)	3·0 (0–11·7)
	IgM	5·1 (0–150)	5·4 (0–35·9)	2·4 (0–15·5)
	IgG	0·3* (0–17·9)	0·0 (0–4·6)	0·0 (0–7·6)
BLG	IgA	8·3* (1·9–53·7)	8·3* (0–46·5)	5·3 (0–29·8)
	IgM	1·0 (0–28·3)	1·2 (0–14·4)	0·8 (0–11·9)
	IgG	0·4 (0–5·8)	0·0 (0–18·8)	0·0 (0–2·9)
OVA	IgA	15·8 (1·9–92·9)	13·2 (0·6–34)	14·3 (3·4–30·2)
	IgM	1·2 (0–34·1)	1·4 (0–16·7)	1·3 (0–13·3)
	IgG	0·0 (0–13)	0·0 (0–3·1)	0·0 (0–1·7)

*Antibody values significantly higher (p<0·05) than in control subjects.
†Antibody values significantly higher (p<0·05) than in treated coeliac disease patients.
BLG = betalactoglobulin; OVA = ovalbumin.

Saliva (Table V)

Salivary antibody values were generally low, with large overlaps beween patient groups. Untreated coeliac disease patients had higher values of IgA and IgG antigliadin antibodies compared with control subjects; only IgA antigliadin antibody values were higher than in the treated patients. Higher values of IgA anti-betalactoglobin antibody were found in both untreated and treated coeliac disease patients compared with control subjects.

SERIAL STUDIES IN COELIAC DISEASE PATIENTS

Seven coeliac disease patients who had a clinical and histological response to a gluten free diet were studied before and after treatment. Serial changes in serum IgA antigliadin antibody and jejunal aspirate IgA and IgM antigliadin antibodies are shown in Figure 3. Whereas serum antibody values fell significantly (p<0·05) with treatment, there was no significant change in the values of intestinal antibody despite histological

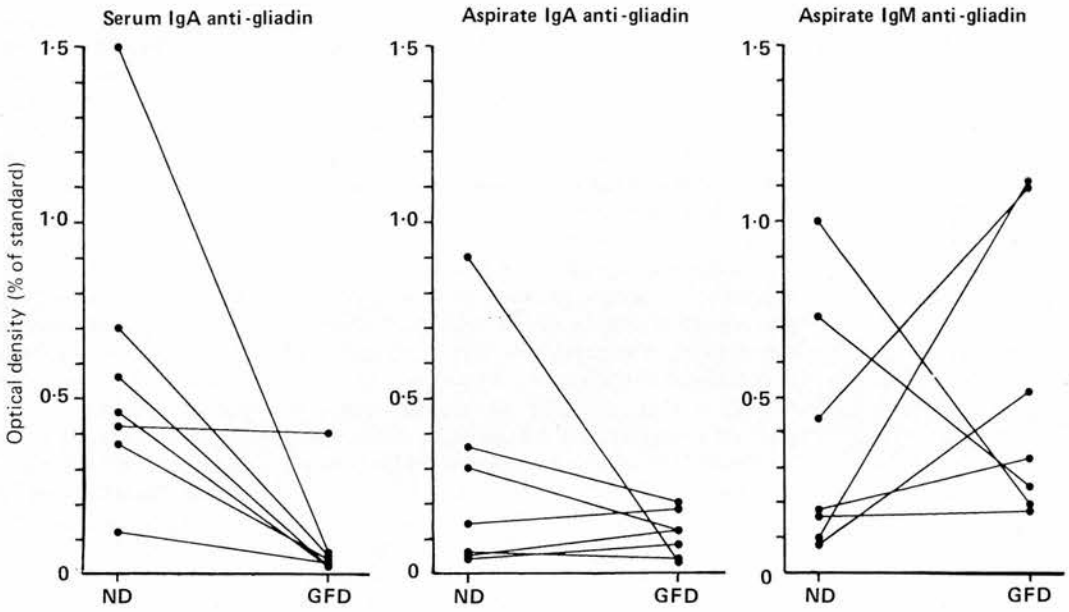

Figure 3: Serial changes in values of serum antigliadin antibody and jejunal aspirate IgA and IgM antigliadin antibodies in 7 coeliac disease patients before and after treatment with gluten free diet. ND=normal diet; GFD=gluten free diet.

recovery on a gluten free diet. There was, in fact, a trend towards higher values of IgM antibody after treatment. It should be noted that these seven patients had been taking a gluten free diet for six months or less, which may account for the fact that they behaved differently from the treated coeliac disease patients as a whole, who had significantly lower jejunal aspirate antibody values compared with untreated coeliac disease patients.

CORRELATIONS BETWEEN ANTIBODY VALUES IN JEJUNAL ASPIRATE, GUT LAVAGE FLUID, SERUM, AND SALIVA (TABLE VI)

IgA antigliadin antibody values were compared in the various body fluids tested. In the untreated coeliac disease patients, we found a positive correlation between antibody values in serum and jejunal aspirate ($r=0{\cdot}68$, $p<0{\cdot}0001$) but in the treated coeliac disease patients this correlation was not maintained as they had high values of jejunal aspirate antibody, whereas serum antibody values were low or absent. In the control group, there was a positive correlation ($r=0{\cdot}59$, $p<0{\cdot}001$) between antibody values in serum and saliva. No correlation was found between salivary and jejunal aspirate antibody values in any of the three groups.

Both jejunal aspirate and gut lavage fluid had been collected from 20 patients (from all three patient groups). A positive correlation ($r=0{\cdot}79$, $p<0{\cdot}01$) was found between antibody values in jejunal aspirate and gut lavage fluid.

Discussion

Intestinal antigliadin antibodies in coeliac disease patients were mainly in the IgA and IgM classes, and significant amounts of IgM antibody persisted in the secretions of treated coeliac disease patients with entirely normal jejunal histology. Secreted IgA antibody was detected only in the subgroup of treated coeliac disease patients with minor histological abnormalities, most of whom had had less than a year's treatment with a gluten free diet. It is possible that the intestinal IgA antigliadin antibody and minor histological changes could both be due to continued ingestion of small amounts of gluten. Conversely, intestinal IgM antibody values remained higher than in control subjects, even in patients with completely normal jejunal mucosa who had been taking a gluten free diet for some years. It is likely that minute amounts of gluten (complete compliance to a gluten free diet is difficult to achieve in adults) maintains a local immune response rather than a systemic one. Our finding of a persistent IgM antibody response parallels the finding of a relatively high fraction of IgM plasma cells in treated coeliac disease patients.[9]

We found high values of serum IgA and IgG antigliadin antibody in untreated coeliac disease patients. In the treated patients, serum IgA antigliadin antibody values were similar to those in controls but serum IgG antigliadin antibody values, though significantly lower than in the untreated coeliac disease patients, remained significantly higher than in control subjects. These findings agree with those of other reports.[17,18]

Concentrations of IgA, IgM, and IgG were all high in jejunal aspirates from untreated coeliac disease patients. Counts of jejunal plasma cells and in vitro immunoglobulin production in coeliac disease are higher than in control subjects for all isotypes,[10] supporting the view that the immunoglobulins (at least IgA and IgM) in coeliac intestinal secretions are produced locally.

TABLE VI *Correlation between IgA antigliadin antibody values in serum, jejunal aspirate, and saliva*

Group	*Serum and saliva*	*Serum and aspirate*	*Saliva and aspirate*
Untreated coeliac disease (n=26)	r=0·021 (NS)	r=0·68 (p<0·0001)	r=0·293 (NS)
Treated coeliac disease (n=22)	r=0·392 (NS)	r=0·04 (NS)	r=0·323 (NS)
Control subjects (n=28)	r=0·593 (p<0·001)	r=−0·193 (NS)	r=0·071 (NS)

NS=not significant.

It is likely that most of the jejunal IgG is plasma derived as the numbers of IgG secreting plasma cells are low even in untreated coeliac disease.[9] We should point out that immunoglobulin measured in jejunal aspirate was total immunoglobulin and not specific secretory IgA and IgM. It is possible that at least some of the jejunal IgA and IgM is serum derived (coeliac disease is a protein losing disorder). We are currently characterising jejunal immunoglobulins and antibody in terms of molecular weight and percentage of total Ig which contains a secretory component. An increase in the intestinal fluid immunoglobulin content was not accompanied by equivalent changes in serum immunoglobulins; in fact, the serum IgM content was noticeably low in some untreated coeliac disease patients.

We have not yet assessed the contribution of specific antigliadin antibody to the increase in intestinal immunoglobulin content in coeliac disease. Falchuk and Strober,[19] using an affinity chromatography technique, reported that approximately half of the net increase in IgA and IgM synthesis (in an in vitro model of gluten challenge) was due to synthesis of antigliadin antibody. Conversely, in a more recent report of in vitro secretion of antigliadin antibody by coeliac jejunal mucosal biopsy specimens,[20] Ciclitera *et al* calculated that antigliadin antibody accounted for 2·1%, 12·1%, and 4·1% of the total concentrations of IgA, IgM, and IgG respectively.

In any event, enhanced intestinal antibody production in coeliac disease is not limited to gluten derived proteins: we found high values of intestinal antibody to betalactoglobulin and ovalbumin in untreated coeliac disease patients, with persistence of IgM antibody to these proteins in treated patients. Antibodies to these food proteins were less specific to coeliac disease than antigliadin antibodies. It has been suggested that high values of serum antibody to these proteins in untreated coeliac disease is simply the result of increased intestinal permeability to antigens.

Although ELISA is now accepted as the standard assay technique for measuring antibodies to food proteins in coeliac disease, several different ELISA methods have been described, and different reference preparations are used by each group of investigators. The method we used is essentially similar to that of Savilahti *et al*,[3] and we used crude gliadin as antigen for the ELISA (rather than say, alphagliadin), as Skerrit *et al*[21] have shown that sera and intestinal aspirates from coeliac patients contain antibodies which bind to several different gliadin subunits. We have not ascribed levels of 'positivity' or 'negativity' to antibody values; such arbitrary designations are of some value in screening tests used in clinical practice but not in prospective research investigations.

Although untreated coeliac disease patients had statistically higher values of salivary IgA and IgG antigliadin antibodies compared with controls, salivary antibodies were generally low with a large overlap between patients and controls. Furthermore, there was no correlation between jejunal aspirate and salivary antibody values. Our results do not suggest that antibody tests on saliva have any diagnostic or screening potential.

On the other hand, gut lavage offers a relatively non-invasive alternative to intubation for the collection of intestinal secretions for immunoglobulin and antibody studies. Antibody findings in gut lavage fluid were broadly similar to those in jejunal aspirate, with a positive correlation for IgA antigliadin antibody values in those patients studied by both techniques. Immunoglobulin concentrations, however, differed considerably in jejunal aspirate and gut lavage fluid; all three immunoglobulin isotypes were raised in jejunal aspirate in the untreated coeliac disease patients, whereas only IgM was significantly raised in gut lavage fluid. Gut lavage fluid is likely to contain secretions from not only the small bowel but also gastric juice, bile, pancreatic secretions, and colonic secretions. In this respect, it is not a homogeneous fluid, unlike jejunal aspirate, which reflects events in the jejunum only, and is thus more likely to represent accurately local immune phenomena in coeliac disease. Fluid flow rate is another factor that may influence Ig values in exocrine secretions; this may partly account for differences in findings in jejunal aspirate and gut lavage fluid.

The relevance of gluten reactive intestinal B cells and antibodies to the pathogenesis of coeliac disease is uncertain; the presence of antibodies to gluten derived proteins in patients with coeliac disease may be merely an epiphenomenon in the context of a T cell mediated enteropathy with expansion of the relevant populations of T helper as well as effector cells.[22] It is certainly possible that mucosal IgM antibody is immunopathogenic, for example by fixing complement in the immediate vicinity of enterocytes.

This study shows the dissociation of systemic and intestinal humoral immune responses in patients with coeliac disease, and is evidence that for the study of immunopathology of intestinal disease, direct investigation of the gut is mandatory.

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