

STUDIES ON THE PATHOGENESIS OF COELIAC DISEASE

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CONTENTS

<u>ABSTRACT</u>	1
<u>INTRODUCTION</u>	3
History	3
Definition of coeliac disease	5
Pathogenesis of the mucosal lesion	8
The search for the toxic fraction	8
Carbohydrate content & toxicity	12
Assessment of gluten toxicity	13
Jejunal mucosal organ culture	15
Mechanism of gluten toxicity	20
The Digestive Enzyme Deficiency Theory	20
The Immunological Theory	23
Evidence relating to the Immunological Theory	
1) Non-specific abnormality of the immune system	24
2) Indirect evidence	31
Animal models	33
Genetic factors & HLA	37
Clinical evidence	43
Associated diseases	43
Organ culture studies	47
3) Specific immunity to gluten	47
Humoral immunity	47
Cellular immunity	55
Problems relating to the LMI test	67
The Lectin Theory	70
Lectins	71
Cell-surface carbohydrates	72
Experimental evidence	75
Phosphatidyl inositol turnover	77
The lectin theory & the LMI test	79

Aims of the Study	81
MATERIALS & METHODS	83
Patients & controls	83
LMI test	84
Antigens	87
Reproducibility of LMI test	91
Influence of HLA & diet on LMI test	92
LMI test with gluten and other dietary antigens	93
LIF production by jejunal mucosa	94
Comparison of B2 and GFIII	96
Effect of rhamnose on LMI test	96
Effect of puromycin on LMI test	97
LMI test using subpopulations of leucocytes	98
Effect of preincubation with serum	102
Jejunal biopsy organ culture studies	103
Jejunal biopsy & lymphocyte co-culture	105
Quantitative histology	106
Biochemical analysis	107
Phosphatidyl inositol turnover	110
RESULTS	117
LMI test - influence of HLA-B8 and diet	117
LMI test with other dietary antigens	119
Comparison of normal and biopsied controls	122
Comparison of B2 and GFIII	122
Effect of rhamnose on LMI test	123
Effect of puromycin on LMI test	123
LMI test using subpopulations of leucocytes	125
Effect of serum incubation on LMI test	127
LIF production by jejunal mucosa	128
Jejunal biopsy and lymphocyte co-culture	129
Phosphatidyl inositol turnover	133

DISCUSSION	134
The LMI test	134
Jejunal biopsy culture studies	155
Conclusions	164
Declaration of Originality	167
Publications arising from this Thesis	168
Acknowledgments	169
REFERENCES	171
Appendix Tables 1 - 7	after page 209

ABSTRACT

Results of the Leucocyte Migration Inhibition (LMI) test with gluten in adult coeliac disease are shown to be affected by the histocompatibility antigen HLA-B8. Normal subjects with B8 show greater immunity than those lacking B8 and give similar results to untreated coeliacs. B8 and non-B8 coeliacs give similar results. A gluten-free diet produces an early increase in immunity in coeliacs with a later decline. Production of migration inhibitory factors in blood and by jejunal mucosa are inversely correlated. Untreated coeliacs give positive responses in the LMI test to several dietary antigens similar in magnitude and prevalence to those seen with gluten. These responses are uninfluenced by HLA-B8 and do not increase after gluten exclusion. Results of the LMI test with gluten are unaffected by puromycin and results of the test performed with purified polymorphs are similar to and correlate with results of the standard test. Added T lymphocytes have no effect. These results contrast with those seen with PPD as antigen. Normal leucocytes can be sensitised to gluten by coeliac serum and this sensitisation can be blocked by IgG, suggesting that migration inhibition is mediated by cytophilic antibody and not T cell produced lymphokines.

Jejunal mucosal biopsies from treated coeliac patients are co-cultured with autologous blood mononuclear cells. These biopsies show no significant damage with gluten or lymphocytes alone but quantitative histology shows evidence of toxicity when biopsies are cultured with gluten and lymphocytes together. Phosphatidyl inositol turnover in cultured biopsies is measured to try and detect a lectin-like effect of gluten but shows no difference between coeliacs and controls.

The findings are discussed and the relevant literature is reviewed.

I N T R O D U C T I O N

"It is a capital mistake to theorize before
one has data"

The Adventures of Sherlock Holmes
Sir Arthur Conan-Doyle, 1891.

History

The word coeliac is derived from the Greek "koiliakos" which means "belonging to the belly; also, suffering in the bowels" (Oxford English Dictionary, 1971). It is perhaps in the latter sense that the word was used by Aretaeus in what seems to be the earliest description of the malabsorption syndrome. Aretaeus is a rather mysterious figure who practised medicine in Cappadocia in Asia Minor in the second century A.D. (Major, 1948). He was thus a contemporary of Galen and in view of his remarkably accurate descriptions of diseases as diverse as diabetes mellitus, epilepsy, asthma, gonorrhoea and elephantiasis it is surprising that his name is not better known. His essay "On the Coeliac Affection" (translated by Francis Adams, 1856) contains a description of steatorrhoea and the effects of malabsorption of food on children and adults. His ideas on therapy are, as one might expect, strange to us. Aretaeus recommends astringent wines, the stones of unripe grapes and digestive medicine made from vipers. However, he also suggests that "the stomach is to be relieved from its suffering by rest and abstinence from food, for in this way the natural powers are restored". At one point, Aretaeus uses the word "siton" for food. A standard Greek-English

dictionary (Liddell & Scott, 1869) defines this word as "food made of wheat or corn" and this, together with Aretaeus' comment that "bread alone contributes little towards strength" raises the possibility that the harmful effects of wheat products on coeliac patients may have been noticed almost two thousand years ago.

The next classic description of coeliac disease was that of Samuel Gee (Gee, 1888). Once more several passages seem strangely perceptive: "The allowance of farinaceous foods must be small", "if the patient can be cured at all it must be by means of diet". Despite these early observations and the use of low carbohydrate diets to treat coeliacs in the early part of this century (Haas, 1932; Sheldon, 1949) it was not until after the Second World War that Dicke, first in his M.D. thesis and then in a series of papers (Dicke, 1950; 1953) demonstrated the dramatic effect of excluding wheat, rye and oats from the diet of coeliac children. At about the same time the pathological lesion of coeliac disease was described by Paulley in specimens of small bowel taken at laparotomy (Paulley, 1954). Within a few years workers on both sides of the Atlantic had devised methods for peroral biopsy of the mucosa of the duodenum and proximal jejunum (Palmer, 1953; Shiner, 1956a; Shiner, 1956b; Crosby, 1957; Brandborg et al. 1959) and the diagnosis of coeliac disease entered the modern era.

Definition of Coeliac Disease

Early definitions of coeliac disease were essentially those of a clinical picture of malabsorption of unknown cause. By the 1950s it was being suggested that it was necessary to demonstrate a raised faecal fat excretion (Cooke, Peeney & Hawkins, 1953) though it was later shown that in fact patients with untreated coeliac disease may have a normal stool fat (Gent & Creamer, 1968). Paulley's description of the typical histological abnormalities (Paulley, 1954) together with the development of techniques for peroral jejunal biopsy have led to much greater precision. The typical histological abnormalities are well known. They consist of:

- 1) Total or almost total loss of villi (Shiner & Doniach, 1960)
- 2) Hypertrophy of the crypts (Rubin et al. 1960a)
- 3) Abnormalities of the epithelial cells: the enterocytes tending towards a cuboidal rather than the normal columnar shape (Yardley et al. 1962)
- 4) Infiltration of the mucosa with lymphoid cells; in particular, an increase in the number of lymphocytes in the epithelial cell layer (Ferguson & Murray, 1971).

In 1967 Pink & Creamer stated that "any condition with a flat jejunal mucosa" constituted the coeliac syndrome and required treatment with a gluten-free diet and Cooke (1968) considered that "the histologically flat jejunal mucosa with mosaic pattern on dissecting microscopy is specific for coeliac disease", confirming earlier ideas that the flat biopsy was diagnostic (Rubin et al. 1960a; Cameron et al. 1962). This view, however, has proved untenable as a wide variety of conditions have been described in which subtotal villous atrophy has been found (see Table 1). Weinstein and his colleagues (Weinstein et al. 1970) proposed two essential criteria:

- 1) A characteristic flat biopsy at the duodenojejunal junction in a patient with malabsorption
- 2) An unequivocal clinical response to a gluten-free diet.

This too has proved unsatisfactory as it is difficult to judge what constitutes an "unequivocal clinical response", especially in those patients who are virtually symptom-free and without steatorrhoea. There is also evidence that gluten exclusion may produce clinical improvement in tropical sprue (Bayless & Swanson, 1964) and regional enteritis (Rudman et al.

TABLE 1

Conditions associated with subtotal villous atrophy
without gluten sensitivity

<u>Condition</u>	<u>Reference</u>
Triparalol therapy	McPherson & Summerskill, 1963
Non-gastrointestinal malignancy	Creamer, 1964
Acute hepatitis	Astaldi et al. 1964
Ulcerative colitis and cirrhosis	Salem et al. 1964
Sarcoidosis	Hindle & Creamer, 1965
Collagen diseases	Siurala et al. 1965
Radiotherapy	Tankel et al. 1965
Lymphoma	Lee, 1966
Uraemia	Loehry & Creamer, 1966
Hypogammaglobulinaemia	Johnson et al. 1967
Zollinger-Ellison syndrome	Shimoda & Rubin, 1968
Tropical sprue	Klipstein, 1968
Kwashiorkor	Brunser et al. 1968
Macroamylasaemia	Levitt et al. 1968
Hypothyroidism	Siurala et al. 1968
Whipple's disease	Maizel et al. 1970
Eosinophilic gastro- enteritis	Leinbach & Rubin, 1970
Collagenous sprue	Weinstein et al. 1970
Tuberculosis	Fung et al. 1970
Giardiasis	Ament & Rubin, 1972a
Soy protein sensitivity	Ament & Rubin, 1972b
Cow's milk protein sensitivity	von Essen et al. 1972
Infective enteritis	Anderson et al. 1972
Small bowel ischaemia	McDonald & Hourihane, 1972
Methyldopa therapy	Shneerson & Gazzard, 1977

1971). The strictest criteria for diagnosis are those laid down by the European Society for Paediatric Gastroenterology (Meeuwisse, 1970) who require a complete restitution to clinical and histological normality with gluten exclusion followed by relapse of the mucosa on gluten challenge. Booth (1974) has proposed a similar definition for adult coeliac disease though without demanding a return to normality on a gluten-free diet.

While these last two definitions seem most suitable for scientific studies of coeliac disease, there are practical problems. Gluten challenge is not without hazard and one death has been reported (Neale, 1968). Booth himself, in discussion, stated that "there is no need to challenge most (adult) patients" (Booth, 1974).

In this study, therefore, patients are accepted as having coeliac disease if the initial jejunal biopsy taken whilst the patient is on a normal gluten-containing diet shows the characteristic abnormalities outlined above and if a subsequent biopsy, taken after a period of gluten exclusion, shows definite histological improvement. A few patients were also accepted as coeliacs who had been taking gluten-free diets for some time without a previous satisfactory

biopsy. These patients were all biopsied whilst on a gluten-free diet, put onto a gluten-containing diet and re-biopsied between three and five months later, or earlier if symptomatic. Clear histological deterioration was accepted as evidence of coeliac disease.

As a final point, it is worth noting that recent studies of high gluten diets in both relatives of coeliac patients and in patients with altered immunity have suggested that significant histological changes can be produced in previously normal mucosa (Doherty & Barry, 1981). Future definitions of coeliac disease may have to be modified to take account of these findings if they are confirmed.

Pathogenesis of the mucosal lesion

The search for the toxic fraction

Once the deleterious effect of wheat on coeliac patients had been established the search for the toxic factor began. Dicke et al. (1953) pointed out that wheat flour was harmful but wheat starch was not and the same workers showed that the toxic factor was found in the protein component and, more specifically, the gliadin fraction of the flour. (van de Kamer et al. 1953). Wheat flour consists of the milled endosperm of the wheat grain. About 75% of the

flour is starch, 7-15% is protein, 1-2% lipid and the remainder is water with variable amounts of hemi-celluloses (Ewart, 1970). Gliadins are separated from the other proteins (albumin, globulin and glutenin) on the basis of being alcohol-soluble and can be separated into α , β and γ gliadins by starch-gel electrophoresis. Alpha-gliadin is the most readily extractable protein and has been widely tested in toxicity studies (Hekkens et al. 1970). It is not, however, a single protein (Bernardin et al. 1967) and seems to be composed of similar polypeptide units to the other gliadins (Patey et al. 1975). Efforts to produce wheat which was non-toxic led to a variety of wheat (multisomic 6A tetrasomic 6D) which produced very little α -gliadin but normal amounts of β and γ gliadin (Kasarda et al. 1978). Sadly, this wheat proved no less deleterious than normal to coeliac patients (Ciclitira et al. 1981).

Frazer et al. (1959) utilised a different approach by performing a peptic-tryptic digest of gluten. It had previously been observed that complete acid hydrolysis of gluten rendered it non-toxic to children with coeliac disease (Alvey et al. 1957; Weijers & van de Kamer, 1958). Frazer and his colleagues stopped their proteolytic digestion short of complete

hydrolysis to produce a water-soluble heterogenous material consisting mainly of polypeptides of molecular weight less than 15,000. This material (the well-known gluten fraction III) was shown to retain the toxicity of untreated gluten by clinical testing. The most extensive series of experiments in search of the toxic fragment have been carried out with gluten fraction III as a starting material and have come from the Oxford group. Dissanayake and colleagues (1974) separated gluten fraction III into three subfractions, A, B and C, on the basis of molecular size by ultrafiltration through a series of membranes. Toxicity was then tested in three coeliac patients and assessed by repeated jejunal biopsy. The fractions containing the larger molecules (B and C) both proved toxic; fraction A, consisting of amino-acids and oligopeptides with molecular weights of under 1,000, was innocuous. Fraction B, consisting of polypeptides with a mean molecular weight of about 8,000, was further separated to subfractions B1, B2 and B3 by gel filtration through a Sephadex G50 column. Feeding experiments showed toxicity residing in the B2 and B3 fractions (Sikora et al. 1976). The B2 fraction had a smaller mean molecular weight than B3, with a mean in the high hundreds (Offord et al. 1978) and as the point of the

experiments has been to identify the smallest peptides which retain toxicity, it is this fraction which has been most used in further studies.

A similar approach to that of the Oxford group, that is proteolytic digestion followed by separation by gel-filtration, has been used by several groups with gliadin rather than gluten as the starting material (Bronstein et al. 1966; Cornell & Townley, 1973a; Hekkens et al. 1974). Hekkens and his colleagues (1974) produced α -GT 18,000 from a tryptic digest and this was found to be toxic in vivo and to react with rabbit anti- α -gliadin serum. Cornell & Townley (1973a) produced the more extensively studied fraction 9 from a peptic-tryptic-pancreatinine digest and this toxic fraction has been further subfractionated by gel-filtration to yield materials of mean molecular weight around 1,500 and which are rather confusingly termed fractions 1B and 2B (Cornell & Rolles, 1978).

Jos and his colleagues (Jos et al., 1982) using gel filtration of digests of β -gliadin have produced a peptide (" β 5") of molecular weight around 7000 shown to be toxic in organ culture studies. Further work on yet smaller fragments of both α -gliadin and B2 has been started (Kasarda et al. 1974; Anand et al. 1977) which includes some preliminary work on amino-acid

sequencing. This may be a little premature as these smaller fractions have not yet been adequately tested for toxicity in vivo, but clearly, if indeed there is a single peptide which causes the mucosal damage in coeliac disease, then it is likely to be identified and characterised within the foreseeable future.

Carbohydrate Content & Toxicity

One group of workers have taken an original and interesting view of the problem of finding the toxic fraction. Phelan and his colleagues from Galway have pointed out that all attempts to abolish the toxicity of gluten by proteolytic enzymes have failed (Phelan et al. 1974). They postulated that the explanation for this fact was not that toxicity resided in a small peptide resistant to proteolysis, but that toxicity may be due to a carbohydrate moiety in the protein. In a series of experiments gliadin was therefore treated with carbohydrases produced from the fungus *Aspergillus niger* and the resulting material was found to be non-toxic in feeding experiments in a small group of coeliac patients (Phelan et al. 1974, 1977, 1978; Stevens, Phelan, McNicholl et al. 1978). Peptide mapping of the treated gliadin showed no differences from untreated gliadin (Phelan et al. 1974) and no evidence of proteolytic activity in the carbohydrases could be found (Phelan et al. 1978).

Fascinating as these results are, it should be emphasised that they have been criticised on the way in which toxicity was assessed with regard to the small numbers of patients used in the feeding experiments, the differences in initial histology between those given treated and untreated gliadin and the duration of the challenge. Furthermore, Bernardin et al. (1976) failed to demonstrate a significant amount of carbohydrate in a gliadin preparation which was toxic. The concept, however, is interesting and far from being disproven, and has relevance to some of the theories of the mechanism of toxicity which are discussed later.

Assessment of Gluten Toxicity

The investigation of the nature of the toxic fraction of gluten, and of the mechanisms by which gluten may be damaging the small bowel mucosa requires a system in which toxicity may be tested and quantified, and in which various pharmacological or immunological modifications may be made to attempt to modify the toxicity shown. Furthermore, such a test system should not involve risk to the health of patients.

As indicated in the previous section, the basic technique used has been feeding of the material under test to coeliac patients and monitoring their

response. Initially this consisted simply of observing clinical status (Dicke et al. 1953) and later by measurements of faecal fat (Fraser et al. 1959). More sophisticated methods of assessing the integrity of absorptive function have been devised and proposed as screening procedures for the diagnosis of coeliac disease, for example xylose absorption (Rolles et al. 1975) and intestinal permeability as assessed by the absorption of two molecules of different sizes (Cobden et al. 1978). The former has been used as a test for gliadin toxicity (Stevens, Watt, Bourke et al. 1978) but has not gained widespread acceptance. As the definition of coeliac disease rests on histological criteria, it seems appropriate to adopt histological methods to test for gluten toxicity and indeed this has been widely done by repeated jejunal biopsies either after a period of gluten feeding (Bayless et al. 1970; Dissanayake et al. 1974; Sikora et al. 1976) or after installation of the material directly into the small bowel or duodenum (Shmerling & Shriver, 1970; Hekkens et al. 1970; Anand et al. 1981).

All these methods of testing in patients, however, involve discomfort to the patient and some minor risk, as gluten challenge may provoke irreversible deterioration (Neale, 1968). Large amounts of test

material are required for in vivo testing and repeated testing and experimental use of pharmacological or immunologically active agents is impractical. Attempts have thus been made to develop in vitro test systems.

Some workers have claimed that fraction 9 of gliadin causes increased acid phosphatase release from rat liver lysosomes (Cornell & Townley, 1973b; Townley et al. 1973) though this has not been confirmed by others using gliadin or Frazer's fraction III (Hekkens, 1978). Cordone et al. (1975) used fibroblasts from coeliac patients as a target and claimed that gluten caused degeneration but, again, this has not been confirmed (Hekkens, 1978). The logical target to use in assessing gluten toxicity is, of course, the jejunal mucosal epithelial cell as this seems to bear the brunt of gluten-induced damage in vivo. As yet there is no satisfactory system for isolating and culturing these cells, but organ culture of fragments of jejunal mucosa is perhaps the next best thing and has proved technically feasible.

Jejunal Mucosal Organ Culture in Coeliac Disease

In 1969 Browning & Trier described a technique with which it was possible to maintain human small intestinal mucosal biopsies in organ culture for 24 hours with good preservation of the mucosal

architecture. The same authors later reported that epithelial cells of biopsies from patients with untreated coeliac disease which were cultured for 24 hours in gluten-free medium showed morphological improvement as assessed by electron microscopy (Trier & Browning, 1970). Subjective improvement in morphology has also been found after culture in gluten-free medium by other workers (Falchuk et al. 1974; Jos et al. 1975; Hauri et al. 1978). Townley et al. (1973), Falchuk et al. (1974) and Jos et al. (1975) all showed that this improvement could be prevented by incorporation into the culture medium of gluten peptides known to be toxic in vivo, though Jos also showed some toxic effect of casein peptides on untreated coeliac biopsies, and Hauri et al. (1978) could demonstrate no in vitro toxicity of gluten fractions shown to be toxic in vivo.

However, all these studies relied on subjective assessment of improvement in histology which may be imprecise. Falchuk et al. (1974), however, also measured mucosal alkaline phosphatase activity in the cultured biopsies and demonstrated a marked rise (from an initially low value) of enzyme activity in untreated coeliac biopsies cultured in the absence of gluten, and abolition of this rise by culture with gluten peptides. Casein peptides had no significant effect.

In 1978 Katz & Falchuk reported use of the technique to predict gluten sensitivity in a group of patients taking a normal diet who had abnormal biopsies, and in 1980 the same group (Falchuk et al. 1980) again showed in vitro gluten sensitivity in untreated or gluten-challenged patients and further suggested that responses in their system depended on histocompatibility type, being most marked in patients having the HLA-B8 antigen, and have also produced interesting results on co-culture of biopsies from treated and untreated patients (Falchuk, Gebhard & Strober, 1974) and on the effect of corticosteroid on the in vitro toxicity of gluten (Katz et al. 1976) which will be discussed in more detail later.

This method of assessing in vitro toxicity has not, however, been unchallenged. Hauri et al. (1978) could show no effect of gluten on brush border enzyme activities (including alkaline phosphatase). Stevens, Keane & Fottrell et al. (1978) and Mitchell et al. (1974) were unable to demonstrate a rise in alkaline phosphatase in biopsies cultured in the absence of gluten and the latter workers also criticised the expression of results in relation to the protein content of the biopsy, as they showed that the protein content per wet weight of the biopsy declined with increasing culture time.

A more recent study (Howdle et al. 1981) has utilised another approach. These workers felt that as the criterion for the diagnosis of coeliac disease is histological then histology is the most appropriate method of assessing in vitro gluten toxicity. Rather than rely on subjective assessment, however, they used objective measurement of enterocyte height as an index of mucosal damage and demonstrated that enterocyte height of biopsies from untreated patients rose during culture in gluten-free medium and that this rise was prevented by gluten fraction III but not by casein. Rapid changes in enterocyte height in vivo have been observed in coeliac disease following dietary modification (Yardley et al. 1962; Bayless et al. 1970). Fluge & Asknes (1981) have produced similar results to Howdle et al. and this method of assessing gluten toxicity in vitro seems likely to prove useful.

Another important finding from these in vitro studies has been the relative insensitivity of biopsies from coeliac patients who had been treated by gluten-exclusion to gluten toxicity in vitro. Falchuk and his colleagues (Falchuk et al. 1974; Falchuk, Gebhard & Strober, 1974) were unable to demonstrate any effect of gluten peptides on treated mucosa in vitro using biochemical assessment, though sensitivity to gluten was

restored by co-culture with an untreated biopsy (Falchuk, Gebhard & Strober, 1974). Fluge & Asknes (1981), using quantitative histology, also failed to show any effect of gluten on treated biopsies, though Howdle et al. (1981) did show a slight but significant decrease in enterocyte height of treated biopsies when cultured with gluten fraction III. This decrease was, however, much less than the changes seen with untreated biopsies. Possible explanations for this difference in sensitivity include activation of some endogenous effector mechanism which is more active in untreated than treated mucosa, and the difference does argue against a direct toxic effect of gluten.

In summary, after over a decade of experience with organ culture studies in coeliac disease, there is still disagreement as to the best method of assessing mucosal damage. Despite this, there is considerable agreement between different groups using different systems that gluten sensitivity can be demonstrated using biopsies from untreated coeliac patients and that this sensitivity is less or absent in biopsies from treated patients. The organ culture system obviously offers great scope for unravelling the mechanisms of gluten toxicity, but apart from the co-culture and steroid experiments of Falchuk and co-workers mentioned

above, there has been little exploitation of the possibility of varying different factors in the system in order to investigate the importance of possible mechanisms of toxicity.

Mechanism of Gluten Toxicity

I. The Digestive Enzyme Deficiency Theory

The suggestion that patients with coeliac disease have an inborn error of metabolism which results in a deficiency of an intestinal digestive enzyme necessary for the complete digestion of gluten dates back to the work of Frazer (1956). Lack of the putative enzyme causes damage to the intestine by allowing the accumulation of a toxic fraction of gluten which in normal people is digested further and rendered harmless. Frazer demonstrated that a peptic-tryptic digest of gluten known to be toxic to coeliac patients in vivo could be made non-toxic by incubation with normal pig intestinal mucosa. Further evidence in favour of this theory has come from studies of the ability of homogenates of intestinal mucosal biopsies from coeliac patients to digest gluten in vitro. An early study of this type (Douglas & Booth, 1970) showed no difference in either the amount of amino-acids liberated or of peptides remaining after incubation with mucosal homogenates from coeliacs or from

controls. Cornell & Townley (1973) repeated this work, however, and by using a smaller fraction as substrate (fraction 9), increasing the mucosal protein to substrate ratio and prolonging the incubation time, they demonstrated a lesser degree of digestion of fraction 9 by coeliac mucosa than by normal mucosa. Residual peptides showed a high content of glutamine, proline and serine and they suggested that a peptidase involved in hydrolysing this combination of amino acids was deficient. Similar results were reported by Carchon et al. (1979) using biopsies from children with coeliac disease. Later work (Cornell & Rolles, 1978) confirmed these findings and also suggested a partial deficiency of the same peptidase in first degree relatives of coeliac patients.

A parallel approach has been to measure intestinal peptidase activity directly. There have been several studies of this type (Messer et al. 1961; Fottrell et al. 1970; Berg et al. 1970; Scott, 1975) which have failed to demonstrate any significant differences between control and treated coeliac mucosa. Earlier work demonstrating lower dipeptidase activity in coeliac mucosa with an improvement following treatment (Lindberg, Norden & Josefsson, 1968) has been criticised as merely representing villous atrophy in

the coeliac patients (Fottrell et al. 1970) and indeed the same criticism can be levelled at any studies comparing coeliac and normal mucosa even if the coeliac mucosa seems normal histologically as enzyme recovery may lag behind histological recovery. More recent work (Sterchi & Woodley, 1978) has shown that peptidases in highly purified brush border membranes from normal human intestinal mucosa have broad specificity and are thus able to digest a range of substrates and suggest that this renders an enzyme deficit in coeliac disease unlikely, though a direct comparison with coeliac mucosa was not made.

Some circumstantial evidence in favour of the enzyme theory comes from observations of early damage to the epithelial cell after gluten challenge (Bayless et al. 1970; Dissanayake et al. 1974) as it is suggested that such damage (within six hours) could not be mediated via immunological mechanisms. This argument, however, is not convincing as immune complex, cytotoxic T cell or antibody-mediated cellular cytotoxic reactions may all be evident in this time scale.

As this thesis offers no evidence directly relating to possible digestive enzyme defects, the topic will not be discussed further. However, the

theory remains with us as (1) it is virtually impossible to disprove and (2) it is not incompatible with the immunological and lectin theories discussed below as the immunogen or lectin implicated in these theories may well represent an abnormal product of digestion.

II. The Immunological Theory

The basic thesis of the immunological theory of the pathogenesis of coeliac disease is that patients with coeliac disease have an exaggerated or aberrant immune reaction to a component of gluten and that this abnormal immune reaction results in damage to the jejunal mucosa.

The small intestine is a major lymphoid organ, which is unsurprising when one considers the enormous antigen load handled by the gut. Numerous alterations of immune function have been observed in coeliac disease. It is, however, difficult to establish whether these various abnormalities have a primary role in causing the mucosal damage or whether they are merely epiphenomena secondary to the mucosal damage.

Evidence relating to the Immunological Theory

1. Non-specific abnormalities of the immune system

A number of immunological changes not particularly concerned with specific immunity to gluten have been described in coeliac disease.

a) Morphological Changes

Several morphological features of coeliac disease have been thought relevant to the immunological theory. These include the increased numbers of lymphoid cells in the lamina propria and epithelial cell layer of the jejunum (Paulley, 1954; Ferguson & Murray, 1971); mesenteric node hypertrophy (Paulley, 1954); peripheral node hypoplasia (McCarthy et al. 1966); splenic atrophy (McCarthy et al. 1966) and the increased incidence of lymphoreticular malignancies in coeliac disease (Harris et al. 1967; Barry & Read, 1973; Holmes et al. 1976; Isaacson, 1980).

b) Changes in Lymphoid Cell Count

Counts of total blood lymphocytes in coeliac disease have given conflicting results (Brandt & Stensham, 1975; O'Donoghue et al.

1976; Bullen & Losowsky, 1977). The number of circulating T lymphocytes, however, does seem to be reduced in untreated coeliacs (O'Donoghue et al. 1976) especially those with hyposplenism (Bullen & Losowsky, 1977), and to rise following gluten withdrawal (O'Donoghue et al. 1976).

The increase in intraepithelial lymphocytes and of plasma cells in the lamina propria of the jejunal mucosa of untreated coeliacs mentioned above has been quantitated by several groups and both parameters return towards normality on gluten withdrawal (Ferguson & Murray, 1971; Lancaster-Smith et al. 1976; Mavromichalis et al. 1976).

It is perhaps worth observing that the assessment of the degree of infiltration of the intestinal epithelium by lymphocytes depends on how the result is expressed. Most studies express lymphocyte counts per 100 epithelial cells and show an increase in lymphocytes in untreated coeliac disease. Marsh (1980) expressed his counts relative to unit area of muscularis mucosae and found decreased lymphocyte counts in untreated

coeliac disease with an increase to normal on gluten withdrawal. He also observed that the lymphocytes in coeliac biopsies were larger and had increased mitotic activity.

Both animal studies (Ropke & Everett, 1976; Guy-Grand et al. 1974) and human studies (Meuwissen et al. 1976) have suggested that most intraepithelial lymphocytes are T cells and recent work suggests that most have the surface markers of suppressor-cytotoxic cells in contrast to lamina propria lymphocytes where less than 40% bear such markers (Selby et al. 1981).

c) Changes in Serum Immunoglobulins

There is a large literature on this topic, which is perhaps surprising as total serum immunoglobulin levels seem unlikely to provide any very helpful information as regards pathogenesis. In general, the findings have been of normal or low IgM and IgG in untreated coeliacs, with a reversion towards normality on treatment by gluten-free diet (Asquith et al. 1969; Baklien et al. 1977). Serum IgE levels seem to be normal, though IgD deficiency has been reported to be common (Asquith et al. 1969).

Perhaps the most notable finding has been the increased incidence of IgA deficiency in coeliac patients (Hobbs & Hepner, 1968; Asquith et al. 1969). Most of these patients seem to respond to gluten withdrawal, unlike patients who have a flat jejunal biopsy with generalised hypogammaglobulinaemia where the relation to gluten is not clear and where chronic infection may be important in producing the mucosal lesion (Ross & Asquith, 1979).

d) Intestinal Immunoglobulin Production

Raised immunoglobulins of IgM, IgG and IgA class have been reported in intestinal secretions of coeliac patients (Hobbs et al. 1969; Asquith, Thompson & Cooke, 1970) but the significance of this is unclear. Results of immunofluorescent counts of lamina propria plasma cells have given conflicting results (Brandtzaeg & Baklien, 1976). In general, there seems to be an increase in IgM and IgG cells which is reduced after gluten withdrawal.

e) Response to Immunisation

Baker et al. (1975) showed impaired primary and secondary antibody responses in coeliacs following immunisation with the bacteriophage ØX174. Switching of IgM to IgG during the secondary response was impaired and the abnormalities shown were more prominent in those patients with hyposplenism. Beale et al. (1971) found normal responses to tetanus toxoid but impaired response to oral polio-virus vaccine. Mawhinney & Love (1975), however, found increased IgA and IgG responses to oral polio vaccine in coeliacs.

These results suggest some alteration in modulation of immune function and may be related to the high prevalence of HLA-B8 in coeliac patients. This will be discussed in more detail later.

f) Complement Changes

Reduced levels of serum complement components C3 and C4 have been found in untreated coeliacs compared with controls (Doe et al. 1974) and gluten challenge of treated coeliacs has been shown to reduce serum C3 within a few hours of the challenge (Doe et

al. 1974) with the appearance of complement breakdown products (McNeish et al. 1974). Gluten fractions have been shown to activate the alternative pathway of complement activation and it has been suggested that this may be pathogenetically important (Massey et al. 1977).

g) Circulating Immune Complexes

Doe, Booth & Brown (1973) reported detection of circulating antigen-antibody complexes in 60% of untreated coeliacs and 10% of treated coeliacs using the Clq precipitation test. Doe, Henry & Booth (1974) reported increased immune complexes following gluten challenge. Mohammed et al. (1976) similarly found a high prevalence of immune complexes in coeliac disease. Later work (Bullen et al. 1980; Ciclitira et al. 1979), however, showed a much lower prevalence of detectable immune complexes which, it was felt, were unlikely to be of pathogenetic significance. More recently still, Hall et al. (1981), using the sensitive Raji cell assay, detected IgA containing complexes in 45% of coeliacs as well as IgG or IgM complexes in 50%.

However, the level of complexes did not correlate with disease activity and was not altered by gluten challenge, again suggesting that complexes may not be a primary phenomenon.

h) Altered cell-mediated immunity

Scott & Losowsky (1976a) reported that only 22% of coeliacs gave positive Mantoux tests compared with 85% of controls, suggesting a general defect in cell mediated immunity. In contrast, Rossipal (1978) showed increased skin reactivity to DNCB in coeliacs compared with controls.

Several groups have used the response to the plant mitogen phytohaemagglutinin (which predominantly stimulates T cells) to assess non-specific cell-mediated immune capability. The most convincing results are again from Scott & Losowsky (1976a) who performed dose-response curves with PHA and demonstrated diminished transformation in untreated coeliacs with improvement after gluten exclusion. There was some evidence that a suppressive serum factor was involved. Coeliac lymphocytes have also been

shown to transform poorly when incubated in a mixed lymphocyte reaction with Burkitt lymphoma cells (Maclaurin et al. 1971), a finding thought to be relevant to the increased incidence of malignancy in coeliac disease.

2. Indirect Evidence

Indirect evidence in favour of immunological mechanisms being involved in the pathogenesis of the mucosal lesion comes from animal models of coeliac disease, from family studies of coeliac disease showing an association with certain histocompatibility antigens, from clinical studies, from the association of coeliac disease with other "immunological" diseases and from some observations made using the jejunal biopsy organ culture system.

Before discussing this evidence, it is worth considering the possible mechanisms by which abnormal immune reactions to gluten might damage the jejunal mucosa. These can be grouped into four main classes of reaction (Coombs & Gell, 1975):

Type I (anaphylactic, reagin dependent). Initiated by antigen reacting with tissue basophils and mast cells which have been passively sensitised with antibody (usually IgE) produced elsewhere, leading to the release

of pharmacologically active substances (vasoactive amines). There is little to implicate this class of reaction in the pathogenesis of coeliac disease.

Type II (cytotoxic). Initiated by antibody reacting either with an antigenic component of the tissue or a hapten linked to the tissue; damage to the tissue is then mediated either by complement activation or via lymphoid cells capable of binding to the Fc portion of the bound antibody. These lymphoid cells are sometimes called K (for killer) cells and the process is referred to as antibody-dependent cellular cytotoxicity (ADCC).

Type III (damage by antigen-antibody complexes). Initiated when antigen reacts with antibody to form complexes which fix and activate complement. The reaction may occur in the tissue spaces where there is local antigen excess (Arthus reaction) or in the blood when excess circulating antigen forms complexes with antibody which are then deposited and activate complement. In both situations the blood vessel walls bear the brunt of the damage and the characteristics of the reaction are oedema, haemorrhage and polymorph infiltration.

Type IV (delayed hypersensitivity, tuberculin type, cell mediated). Initiated by interaction of antigen with T lymphocytes specifically reactive to that

antigen and which respond by releasing soluble substances (lymphokines) which may be cytotoxic or modulate the local immune response, and/or by becoming cytotoxic. The reaction is characterised by its delayed time course (12-48 hours after challenge) and by infiltration of mononuclear cells, most of which are macrophages.

In the subsequent discussion, it is necessary to bear in mind that these are artificial distinctions and that in vivo it is extremely unlikely that one of the above mechanisms (with the possible exception of IgE-mediated responses) is ever acting alone, and that different mechanisms may predominate at different stages in the complete time-course of a hypersensitivity reaction.

a) Animal models of coeliac disease

There are a number of naturally occurring diseases in animals which resemble coeliac disease to a greater or lesser extent. In addition, various drugs and chemicals have been shown to cause villous atrophy (see review by Haeney, Ferguson & Asquith, 1979). The condition from which most has been learned is *Nippostrongylus braziliensis* infestation in rats. This intestinal

nematode causes patchy small intestinal villous atrophy and crypt hyperplasia with increased epithelial cell turnover (Symons, 1965), enzyme changes (Symons & Fairburn, 1963) and malabsorption (Symons et al. 1971). The infection is normally self-limited and self-cure begins at around the eleventh day. Expulsion of the worms has been shown to require both humoral and cellular immune responses (Dineen et al. 1973). Ferguson & Jarrett (1975), in an elegant series of experiments, showed that thymectomised rats infected with *N. braziliensis* both failed to eliminate the worms and largely escaped the intestinal damage, suggesting that the villous atrophy and crypt hyperplasia were secondary to T cell-mediated immune reaction to helminth antigens. Similar conclusions have been drawn from experiments involving allograft rejection of small intestine (Ferguson & Parrott, 1972, 1973; MacDonald & Ferguson, 1976). Using foetal mouse intestine (which is sterile, thus avoiding complications in interpretation of pathological changes)

grafted under the renal capsule of normal adult mice, they showed that rejection of the graft was characterised by lymphocyte infiltration of the lamina propria and then of the epithelium, with villous atrophy. Rejection in thymectomised recipients was delayed, showing the process to be thymus dependent (Ferguson & Parrott, 1973). The histology thus resembled that of coeliac disease, though the enterocytes were unaffected until late in the rejection process.

Graft-versus-host (GvH) disease is another T-cell mediated condition in which the small intestinal changes resemble those of coeliac disease (though with less cellular infiltration). Reilly & Kirsner (1965) induced GvH disease in F_1 hybrid mice injected with parental spleen cells. The mice developed severe runting with small intestinal changes reminiscent of coeliac disease though with pathological changes more severe in the ileum than in the jejunum. GvH-like reactions have been proposed as a possible mechanism of mucosal damage in

coeliac disease (Neild, 1981) though this hypothesis is rather confused as no explanation is given for how the gut lymphoid cells to which the gluten supposedly becomes attached are thus rendered able to recognise other, unaltered, cells in the gut as foreign and mount an attack on them.

Finally, Bicks and his colleagues (Bicks et al. 1967; Bicks, Azar & Rosenberg, 1967) sensitised guinea-pigs to dinitrochlorobenzene (DNCB) (a potent skin sensitiser, reaction to which seems to be T cell mediated) and then applied DNCB in an alkaline base (Orobace) to colonic mucosa and, by feeding, to small bowel mucosa. In the former case, lesions reminiscent of ulcerative colitis were produced and, in the latter, lesions resembling those of coeliac disease with evidence of xylose malabsorption.

The above experiments seem to establish that T cell mediated immune responses in the gut are capable of causing lesions similar to those seen in coeliac disease. They should not, however, be interpreted as meaning that

only T cell reactions can cause such lesions. It is probable that the small intestine, in common with many other organs, has a limited number of responses to any insult and that morphologically similar damage can result from a wide variety of noxious influences.

b) Genetic factors in coeliac disease and the HLA system

An increased prevalence of coeliac disease in relatives of coeliac patients has been suspected since the 1920s. A series of studies of first degree relatives has given a mean frequency of coeliac disease of 13.5% (Ellis, 1980), though in some studies selection of relatives for jejunal biopsy was not random. It rapidly became apparent that the inheritance of the disease could not be explained on the basis of a simple autosomal recessive or sex-linked gene, though dominant inheritance with incomplete penetrance or polygenic inheritance remain possible mechanisms. That genetic factors are not the entire explanation is shown by studies of

monozygotic twins who are discordant for the disease (Hoffman et al. 1966; Walker-Smith, 1973).

The most important observation in this field has been that of the greatly increased prevalence of the histocompatibility antigen HLA-B8 in patients with coeliac disease compared with controls (Stokes et al. 1972; Falchuk et al. 1972; Harms et al. 1974). About 80% of coeliac patients bear this antigen compared with only around 20% of the normal population. An even stronger association has been reported with the D locus antigen DW3 and the very closely related or identical B cell alloantigen DRW3 (Keuning et al. 1976; Ek et al. 1978) as well as with another, probably distinct, B cell antigen (Mann et al. 1976). Family studies have shown that in general within families, coeliac disease segregates with HLA-B8 (Stokes et al. 1973; Harms et al. 1974) and that affected members of a family usually share HLA antigens (Falchuk et al. 1978). Affected siblings are very rarely, if ever, totally HLA non-identical (Falchuk et al. 1978; Ellis, 1980).

The relevance of the above studies to the immunological theory rests with the possible association of genes determining the histocompatibility antigens with genes controlling immune responses. The HLA complex represents the human major histocompatibility antigen system and is the equivalent of the H-2 complex in the mouse (for reviews see Festenstein & Dement, 1978; Benacerraf & Unanue, 1980). The HLA system consists of four major genetic loci designated HLA-A, HLA-B, HLA-C and HLA-D. Typing for the A, B and C antigens is performed by cytotoxicity testing using antisera. The antigens of the D locus seem to be analogous to the Ia antigens of the mouse and are expressed only on certain cells, notably B lymphocytes and macrophages; D locus antigens are detected principally by analysis of T cell reactivity in mixed lymphocyte reactions - an in vitro model of the recognition phase of allograft rejection. The D related (DR) antigens are closely linked to, and may be identical with, the D antigens but are detected serologically.

The HLA loci are all located on the short arm of chromosome 6 and are closely linked i.e. are physically close and recombination between loci is uncommon. Each HLA haplotype (the specific combination of the alleles at all loci of the histocompatibility complex) is transmitted as a single Mendelian co-dominant trait. In addition to close linkage, the HLA system shows several examples of linkage disequilibrium, that is the phenomenon of certain haplotypes occurring more often than would be expected from the population frequencies of the specific alleles making up the haplotype. In the context of coeliac disease, the known linkage disequilibrium between HLA-A1 and HLA-B8 has been used to explain the increased prevalence of the former in coeliac patients. Similarly, HLA B8 and HLA-DW3 are in strong linkage disequilibrium in the normal population (Keuning et al. 1975) and it seems likely that DW3 is more closely associated with the "coeliac disease gene" and that B8 is merely another marker for this gene, without being primarily involved in pathogenesis (Strober, 1980).

There is considerable evidence in several species of animal that immune response (Ir) genes which modulate the immune response to specific antigens exist and are commonly linked to the genes controlling the major histocompatibility complex (Benacerraf & McDevitt, 1972; Balner, 1976; Balcarova et al. 1974; Landolfo et al. 1978). In man, HLA status has been shown to affect immune responses to several antigens, including streptococcal antigens (Greenberg et al. 1975), influenza vaccination (Spencer et al. 1976), vaccinia virus (de Vries et al. 1977), tetanus toxoid (Sasazuki et al. 1978), human liver specific lipoprotein (Vogten et al. 1979), schistosomal worm antigens (Sasazuki, Otta, Kaneoka et al. 1980; Sasazuki, Kaneoka, Nishimura et al. 1980) and ragweed allergens (Levine et al. 1972). Of particular interest are the reported immunological effects associated with possession of HLA-B8. These include a generalised increase in immune reactivity in the mixed lymphocyte reaction (Osaba & Falk, 1978), increased cell mediated immunity to liver specific lipoprotein (in

patients with severe liver disease) (Vogten et al. 1979) and increased antibody titres to rubella, measles and some autoantibodies (again in patients with chronic liver disease) (Galbraith et al. 1976). In addition, there is evidence of increased immune reactivity to gluten in HLA-B8 individuals without coeliac disease, both on the basis of increased gluten antibodies (Scott et al. 1974) and increased lymphocyte transformation (Cunningham-Rundles et al. 1978). This is further discussed below. Finally, HLA-B8 has been associated with decreased cell mediated immunity in scleroderma patients (Kellenburg et al. 1981) and a functional Fc-receptor deficit on T cells (Lawley et al. 1981).

Coeliac disease is thus associated with HLA antigens which themselves are associated with a variety of effects on the immune system. It is tempting, therefore, to conclude that these alterations in immune responses may be implicated in the pathogenesis of coeliac disease. Other explanations are, however, available. Histocompatibility antigens are

cell-surface components and the toxic component of gluten may damage cells by binding to these substances, the associated immune phenomena being irrelevant.

c) Clinical evidence

Two clinical observations have been thought pertinent to the immunological theory: (i) the occurrence of "gliadin shock" - a reaction reminiscent of an acute allergic reaction sometimes occurring in coeliac children on a gluten-free diet who are exposed to small amounts of gluten (Krainick et al. 1958) and (ii) the improvement in the jejunal mucosa of coeliac patients seen when they are treated with corticosteroids without gluten exclusion from the diet (Wall et al. 1970).

d) Associated diseases

There is a large number of reports of coeliac disease occurring in association with other diseases thought to have an immunological basis (see Table 2) and from this it is argued that immunological mechanisms are likely to be important in coeliac disease (Lancaster-Smith et al. 1974). Dermatitis

TABLE 2

"Immunological" diseases associated with coeliac disease

<u>Disease</u>	<u>References</u>
Endocrine: Thyroid disease	Green & Wollaeger,1960
Diabetes mellitus	Green & Wollaeger,1960
Addison's disease	Booth,1970
Infertility (?)	Farthing et al.1982
Gastrointestinal:	
Chronic liver disease	Green & Wollaeger,1960
Inflammatory bowel disease	Kitis et al.1980
Rheumatic: Systemic lupus erythematosus	Siurala et al.1965
Rheumatoid disease	Lancaster-Smith et al.1974
Sjogren's syndrome	Lancaster-Smith & Strickland,1971
Polyarteritis and undefined collagen disease	Siurala et al.1965
Pulmonary: Fibrosing alveolitis	Lancaster-Smith et al.1971
Bird-fancier's lung	Berrill et al.1975
Farmer's lung	Robinson,1976
Sarcoidosis	Scadding,1970
Idiopathic pulmonary haemosiderosis	Wright et al.1981
Asthma	Hodgson et al.1976
Dermatological:	
Cutaneous vasculitis	Doe et al.1972
Vitiligo	Lancaster-Smith et al.1974
Eczema	Cooper et al.1978
Dermatitis herpetiformis	Marks et al.1966
Neurological:	
Epilepsy	Chapman et al.1978
Polyneuropathy & myopathy	Berrier et al.1978
Cardiac: Recurrent pericarditis	Dawes & Atherton,1981
Renal: Glomerulonephritis	Swarbrick et al.1980

herpetiformis is the condition most obviously related to coeliac disease as it has a similar relationship to HLA-B8 (Keuning et al. 1976), the majority of the patients have a coeliac-like lesion of the proximal small bowel (Brow et al. 1971) and both skin and bowel lesions seem to respond to gluten withdrawal (Fry et al. 1973). The skin contains deposits of IgA and complement at the dermo-epithelial junction (Seah et al. 1972) and it seems likely that these may represent deposited immune complexes originating from the gut (Scott & Losowsky, 1975). The association with other diseases has also been suggested to be secondary to circulating immune complexes (Scott & Losowsky, 1975) though the evidence for this is slender.

Several of the conditions listed in Table 2 are known to be more common in individuals who have HLA-B8 (Svejgaard et al. 1975) and the association with coeliac disease may be secondary to the association with B8. Overall, however, the evidence does seem in favour of some tendency towards immunological

disturbance uniting these assorted diseases, though it is worth noting that the diagnosis of coeliac disease was not made strictly in some earlier reports and that some of the associations listed are only documented by isolated case reports.

One group of diseases worthy of further discussion is the chronic lung fibroses associated with coeliac disease, and in particular bird-fancier's lung. This is because of the interesting observations from Oxford (Hendrick et al. 1978; Faux et al. 1978) suggesting that coeliacs commonly have circulating antibodies to avian antigens without ever having kept birds and that the antigen involved is a component of egg yolk and is derived from the diet. This raises the interesting possibility that these patients have lung disease not because of inhalation of avian antigens but because of abnormal absorption of antigen from leaky gut mucosa, supporting earlier theories explaining disease associations (Scott & Losowsky, 1975).

Also of interest is the observation that coeliacs have a high prevalence of autoantibodies compared with normal subjects (Seah et al. 1971; Lancaster-Smith & Strickland, 1971; Hodgson et al. 1976; Williamson et al. 1976). One of these antibodies is the well known reticulin antibody (Seah et al. 1971; Alp & Wright, 1971; von Essen et al. 1972; Brown et al. 1973; Rizzetto & Doniach, 1973). This is found in a high proportion of untreated coeliacs and seems to decline following gluten withdrawal (Alp & Wright, 1971; von Essen et al. 1972) but is also found in a quarter of Crohn's disease patients (Alp & Wright, 1971) and in many other conditions (Rizzetto & Doniach, 1973). Early suggestions that the antigen involved was cross-reactive with gluten have not been confirmed (Brown et al. 1973) and it seems probable that the antibody either reflects non-specific tissue damage or, perhaps more likely, a response to dietary antigens other than gluten (von Essen et al. 1972).

e) Organ culture studies

Both cortisol (Katz et al. 1976) and the topical steroid clobetasone butyrate (Bramble et al. 1981) have been shown to diminish the toxicity of gluten to coeliac biopsies in organ culture, thus reproducing the in vivo effect of steroids in coeliac disease discussed above. One possible explanation is, of course, that steroids exert this effect through their immunosuppressive actions.

3. Specific immunity to gluten

Detection of specific immunity to gluten in coeliac disease is obviously central to the immunological theory. There is evidence of both humoral and cell mediated immunity to gluten. The difficulty, as always, is deciding whether this immunity is pathogenetically important or merely an epiphenomenon.

a) Humoral immunity

Circulating antibodies to gluten products in coeliac disease have been known for 20 years (Taylor et al. 1961). Taylor and his colleagues using a tanned red cell haemagglutination technique, showed significantly

higher titres of anti-gluten antibodies (using gluten fraction III as antigen) in both children and adults with coeliac disease compared with normal controls. These results have been confirmed many times using various techniques and various fractions of gluten as antigen (Kivel et al. 1964; Alarcon-Segovia et al. 1964; Katz et al. 1968; Kenrick & Walker-Smith, 1970; Ferguson & Carswell, 1972; Kumar et al. 1976; Stern et al. 1979a, 1979b). Stern and his colleagues, using an immunofluorescent technique, showed that the bulk of their anti-gliadin antibodies were of IgG class whilst 27% of coeliac children had IgA antibody in addition (Stern et al. 1979b).

In general, circulating antibodies to gluten have been more easily detected in coeliacs on a normal diet and titres are lower after a period of gluten exclusion and do seem to correlate reasonably with jejunal histology (Kumar et al. 1976; Stern et al. 1979a, 1979b). Sequential studies have been performed on only a few patients but the results are slightly paradoxical. Rossipal

(1974) showed that in three coeliac children antigluten antibody titres rose in the first few months of a gluten free diet. Scott (1975) found a similar pattern in four of eight adult coeliacs and Stern et al. (1979a) similar results in three of four children tested in the first three months of gluten exclusion. Stern et al. also reported paradoxical decreases in titres after gluten challenge. These results have been interpreted as supporting a pathogenetic role for gluten antibody as it is argued that antibody in the gut which normally complexes with gluten antigen to cause damage is free to diffuse into the circulation when antigen load is reduced (Scott, 1975).

There has, however, been doubt as to the importance of gluten antibodies in pathogenesis since the early reports of their existence. Firstly, coeliac patients also have abnormally high titres of circulating antibodies to dietary antigens other than gluten. These include milk antigens, egg antigens, antigens from cereals other than wheat and sera from a variety of animal

sources (Taylor et al. 1964; Kivel et al. 1964; Ferguson & Carswell, 1972; Stern et al. 1979b). Some of the gluten antibodies are to components of gluten known to be harmless (Stern et al. 1979b). Secondly, antibodies to gluten are found in other gastrointestinal diseases such as aphthous ulceration, pernicious anaemia, duodenal ulceration, ulcerative colitis (Taylor et al. 1964) and in a variety of malabsorptive disorders in children (Stern et al. 1979a), as well as in a few normal controls (Taylor et al. 1964). Though data are very scanty, it seems that titres of antibodies to dietary antigens other than gluten fall after gluten exclusion without an initial rise (Scott, 1975; Kumar et al. 1976).

Antibodies to gluten have also been found in the intestinal secretions of coeliacs but are similarly non-specific both with regard to antigen and disease (Ferguson & Carswell, 1972).

Results of studies on anti-gluten antibody production by cultured jejunal mucosa are conflicting. Falchuk & Strober (1974) showed

that coeliac mucosa challenged in vitro with gluten responded with a marked increase in synthesis of IgA and IgM antibodies and that half of the synthesised antibody had anti-gliadin specificity. Brandzaeg & Baklien (1976), however, using immunohistochemical techniques, found that only 5.7% of IgG cells and 1.6% of IgA cells in a biopsy from an untreated coeliac were producing antibodies to gluten, whilst no antibody to gluten was detected in several other untreated coeliac biopsies.

The relation of anti-gluten antibodies to HLA-B8 status has been investigated by Scott et al. (1974). These workers found that coeliac patients, whether B8 or not, tended to have high titres of gluten antibodies. However, in a group of non-coeliac controls, including patients with chronic liver disease who had high titres of antigluten antibody, the antibody titre was clearly affected by HLA-B8, being higher in the group having the B8 antigen. It was suggested that this may reflect a specific immune response gene for gluten in linkage disequilibrium with HLA-B8. It is of interest that humoral responses to gliadin in the mouse have also



been shown to be linked with the major histocompatibility complex (Johnson et al. 1980).

Though the importance of humoral immunity to gluten has been questioned on the grounds outlined above, support for its possible importance has come from clinical studies involving (i) skin tests with gluten subfractions (ii) the site, timing and nature of the intestinal lesion seen after gluten challenge and (iii) some in vitro studies.

(i) Skin testing with gluten

Early results of skin tests with relatively crude gluten preparations gave generally negative results (Collins-Williams & Ebbs, 1954; Alvey et al. 1957; Breton et al. 1959; Housley et al. 1969). Asquith (1974) showed histological changes of a mononuclear cell infiltrate after skin testing with gluten fraction III, but these occurred equally in coeliacs and controls and so cannot be considered relevant. Baker & Read (1976), using a crude peptic-tryptic digest of

gluten found positive skin reactions in 50% of untreated coeliacs and about 20% of treated coeliacs with no false positives in normal controls. This reaction was maximal at 5-8 hours, was correlated with the presence of serum gluten antibodies and histologically consisted of an infiltrate of polymorphs and mononuclear cells - all features of an Arthus (type III) reaction. Anand et al. (1977) reported a similar time course for skin reactions to fraction B2 and to gluten fraction III, with 100% of coeliacs responding to B2. There has been no real evidence of either immediate (type I) or delayed (type IV) hypersensitivity to gluten in any study.

(ii) Site and timing of mucosal damage after gluten challenge

Studies on this problem have produced differing results. Dissanayake et al. (1974) found epithelial cell damage preceding changes in the

basement membrane and capillaries, whilst Shiner (1973) found the earliest changes in the subepithelial tissues. In general, changes have been found within a few hours of challenge. Anand et al. (1981) not only confirmed that the timing of the damage was consistent with an Arthus reaction but that the histology was also compatible, though immunofluorescence for complement deposition was negative. This last finding disagrees with earlier studies where there was evidence of immune complex and complement deposition in the mucosa (Shiner & Ballard, 1972; Doe et al. 1974).

(iii) In vitro studies

In contrast to the above work which is, overall, in favour of a type III reaction to gluten, Ezeoke and his colleagues (Ezeoke et al. 1974) have shown evidence of a type II reaction to gluten by demonstrating that coeliac sera commonly contain anti-

bodies capable of co-opting K cells to attack various gluten-labelled targets, including normal human jejunum. Although these studies can be criticised on the basis of crude methods of organ culture and possible irrelevance of the other targets chosen (chicken red cells and cellulose particles), the results are convincing.

b) Cellular immunity

In vitro measurement of cell mediated immunity is a difficult subject. There are essentially three types of assay of which two have been used in the context of coeliac disease.

(i) Cytolytic T cell assays Assays for cytolytic action of T cells provide perhaps the most direct and understandable assessment of cell mediated immunity. They are generally performed by labelling target cells with ^{51}Cr and measuring cytolysis by isotope release on exposure of the target to T cells. The assay is only

applicable when cell-surface antigens are involved and has not been used in coeliac disease.

(ii) Lymphocyte transformation assays

Lymphocytes exposed to antigen to which they are sensitised react by blast transformation which can be measured in a variety of ways, but is now most often done by measuring the incorporation of labelled thymidine into DNA produced by the transformed cells. The responding cells probably belong to a number of T cell sub-populations and may also include B cells.

This assay has been extensively used in coeliac disease, but with somewhat conflicting results (see review by Asquith & Haeney, 1979). Early studies failed to show transformation of peripheral blood lymphocytes of coeliacs in response to gluten (Housley et al. 1969; Ansaldi et al. 1970; Morganroth et al. 1972) though mesenteric node lymphocytes did respond (Housley et al. 1969).

Other work, however, has suggested that peripheral blood lymphocytes may show a (usually slight) reaction to gluten fractions in a proportion of coeliacs (Asquith, Housley & Cooke, 1970; von Bulow et al. 1974; Scott, 1975; Holmes et al. 1976; Sikora et al. 1976). This proportion is greater in coeliacs on a gluten free diet than in those on a normal diet and it has been suggested that this represents trapping of gluten-reactive lymphocytes in the gut by gluten antigen in a normal diet. Once gluten is excluded these cells are free to join the recirculating pool (Holmes et al. 1976).

These studies are attractive but are open to criticism. The amounts of antigen used have been very large (up to 4mg/ml), and the changes seen slight. Expression of results has generally been as transformation ratios which may be misleading (Haeney & Asquith, 1976). In at least one

study (Sikora et al. 1976), which claimed to show significant transformation to fraction B2 in a high proportion of coeliacs, peak responses were seen at 48 hours - a time course much more in favour of non-specific lymphocyte stimulation (as by lectins) than a specific antigen-induced reaction, where maximal response is generally seen at five to seven days (Valentine, 1971; Waithe & Hirschhorn, 1973).

By analogy with the humoral responses to gluten, it is clear that it is necessary to show cell mediated reactions to gluten in coeliac disease are specific to gluten by testing other dietary antigens. This has not been adequately done, though Asquith et al. (1970) reported essentially negative results with egg albumin and casein in a small group of patients. However, Jos (1974) has found transformation with milk protein in some coeliac children without overt

milk sensitivity, though no experimental details were given. Finally, there is a single report of increased lymphocyte transformation in three of 15 coeliacs with an antigen consisting of a crude freeze-dried extract of normal human jejunal mucosa (Scott & Losowsky, 1976b). The significance of this is not clear, but two of the three responders were suffering from the relatively rare complication of small bowel ulceration, and the response may have particular relevance to this complication.

(iii) Lymphokine assays

One of the features of cell mediated immune reactions is the liberation of soluble mediators by T lymphocytes when they are exposed to an antigen to which they are specifically reactive. These mediators are known as lymphokines (Dumonde et al. 1969). Measurement of lymphokine activity has thus been used as a measure of the

degree of cell mediated immunity to various antigens.

Lymphokines are identified by their biological activity and have not been fully characterised. Biological activities of lymphokines include migration inhibition factors for both macrophages and leucocytes, skin-reactive factor, transfer factor, various chemotactic factors, factors stimulating lymphocyte transformation, macrophage-activating factor, factors affecting vascular permeability, factors decreasing leucocyte "stickiness" and interferon (Sell, 1980). Of these, the factors affecting leucocyte adherence and migration inhibition have been studied in coeliac disease.

(a) Leucocyte adherence inhibition

Two groups have reported a decrease in leucocyte adherence when mixed leucocytes from coeliac patients were exposed to gluten fractions (Allardyce & Shearman, 1975; Stosiek & Varga,

1980). Allardyce & Shearman did comment, however, that the T cell mediation of this test was not established and that, using PPD, they had evidence that other factors were involved. It is fair to comment that this test is difficult to quantitate and that aberrant results and the involvement of serum factors have been reported, even using antigens such as PPD (Bullen & Losowsky, 1978a).

(b) Migration inhibition tests There are two ways in which migration inhibition tests can be performed. In the indirect assay lymphocytes are cultured with the antigen and the migration inhibitory activity of cell-free supernate is then assessed on whatever target cell is chosen (guinea-pig peritoneal macrophages or normal human leucocytes being the commonest). In the direct assay, mixed blood leucocytes are cultured in the presence and absence of antigen and their migration is compared.

Inhibition of migration in the presence of antigen is a positive response. Migration is measured as the area over which cells spread having either been packed into capillary tubes or incorporated into sloppy agar droplets. Results of these last two techniques correlate closely (Adelman et al. 1980). An example of the capillary tube method, with gluten fraction III as antigen, is shown in Figure 1.

The first results using the Leucocyte Migration Inhibition (LMI) test in coeliac disease came from Ferguson et al. (1975). These workers used a variation of the indirect test by incubating fragments of jejunal mucosal biopsies with or without the addition of α -gliadin. Culture medium from biopsies from coeliac patients cultured with α -gliadin was shown to inhibit the migration of blood leucocytes from healthy controls. These results have been confirmed by

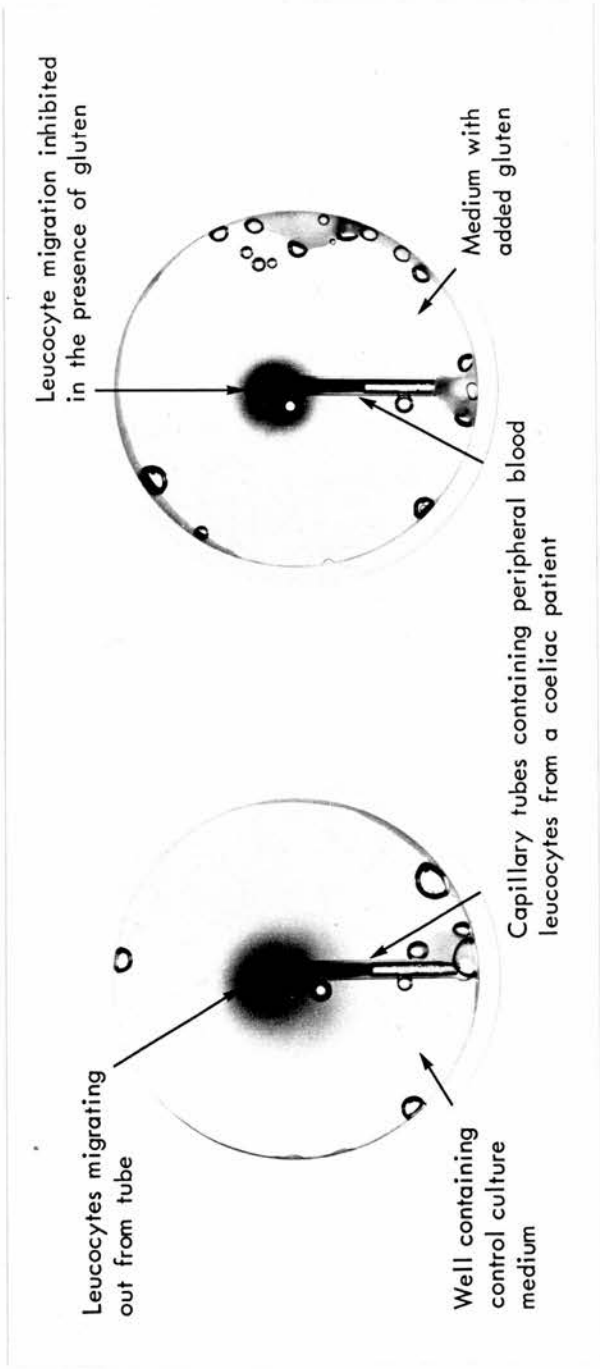


FIG. 1. The capillary tube direct Leucocyte Migration Inhibition test

Howdle et al. (1979) who also observed that the inhibition was greater with medium from biopsies of untreated coeliacs than from coeliacs treated with a gluten-free diet.

Douwes (1976) reported use of the direct LMI test with peripheral blood leucocytes in coeliac disease. Using an unspecified gluten subfraction, he demonstrated leucocyte migration inhibition in 100% of his coeliac patients and in none of his controls, either healthy or suffering from other gastrointestinal disease. The patients' dietary status was not given.

Bullen & Losowsky (1978b) reported less dramatic results with gluten fraction III. As a group, untreated coeliacs showed significant inhibition of leucocyte migration with gluten, but only 13% gave results outside the normal range. Patients on a gluten free diet, however, showed more marked inhibition with 54% outside the normal

range, i.e. showing definite sensitisation. In treated patients, patients with raised interepithelial lymphocyte counts in the jejunal mucosa showed more migration inhibition. These results were felt to support the suggestion of Holmes et al. (1976) relating to sequestration of gluten-reactive lymphocytes in the gut by dietary gluten.

Askenazi et al. (1978) suggested that the LMI test could be used diagnostically. Using their own B2 and B3 subfractions at a very low concentration (conventionally high antigen concentrations are used in the direct LMI test) they found that 96% of their coeliac patients gave a positive response to one or other antigen compared with only 6% of controls. Controls were said to react only to B2 or B3, never to both, and reactivity of coeliac leucocytes was unaffected by dietary status. This study is open to criticism, however,

as the results were expressed as percentage migration inhibition rather than as a migration index (the ratio of areas of migration in the presence and absence of antigen). Controls in whom the migration index was greater than one (i.e. those whose leucocytes migrated more in the presence of gluten than in control medium) were shown as having 0% migration inhibition. Parametric statistics were then used to calculate a normal range when the results from the control group were clearly non-Gaussian in distribution. Despite this, however, separation between the groups was impressive.

Haeney & Asquith (1978), using α -gliadin, reported results very much like those of Bullen & Losowsky (1978b) and also used α -lactalbumin as a control dietary antigen with negative results. They also found that treated coeliacs gave more positive results with the gluten

antigen than did untreated coeliacs. Ashkenazi et al. (1980) attempted to explain the discrepancy between their results and those of other workers. Using gluten fraction III in their system, they obtained results reasonably comparable with other reported results but still much less impressive than their B2 or B3 results. They also found that mixing B2 and B3 decreased responses and suggested some form of masking of antigenic sites on the molecules when mixed, though a mechanism for this is a little difficult to envisage. The same group (Ashkenazi et al. 1981) have recently revised their previous opinion and agreed that gluten exclusion seem to increase migration inhibition by gluten of peripheral blood leucocytes.

Finally, O'Farrelly et al. (1981) have used the indirect LMI test on peripheral blood leucocytes and find that 100% of untreated and 93% of treated coeliacs give positive results with very small amounts of α -gliadin.

Problems relating to the LMI test and cellular immunity to gluten

There are several aspects of the use of the LMI test and the role of cell-mediated immunity in coeliac disease which remain controversial. First, there is the disagreement as to the diagnostic power of the direct LMI test (there is as yet insufficient data on the indirect test to assess its value). One possible explanation is that proposed by Ashkenazy, which is that their B2 subfraction is much superior to cruder gluten preparations and this gives better results at much lower antigen concentrations. However, B2 and the cruder fractions have only been directly compared by Ashkenazy et al. in their agar drop system (Ashkenazy et al. 1980) and then only with concentrations of gluten fraction III known to be sub-optimal in the capillary tube system (Bullen & Losowsky, 1978).

Second, the consensus now is that cellular sensitivity to gluten, whether detected by the LMI test or by transformation studies, is more easily detected in the blood of patients on a gluten-free diet and in biopsies of patients on a normal diet. This is explained by the shift of committed lymphocytes proposed by Holmes et al. (1976). However,

simultaneous studies of reactivity of blood cells and biopsies have not been performed and the effects of gluten exclusion for varying lengths of time have not been formally studied. Related to this problem is that of the relative insensitivity of biopsies from treated coeliac patients to the toxic effect of gluten in vitro and the resensitisation conferred by co-culture of such biopsies with "untreated" biopsies (Falchuk, Gebhard & Strober, 1974). This seems to tie in nicely with the concept that treated biopsies have insufficient effector lymphoid cells left in them to render them sensitive to gluten (assuming that the lymphoid cells or their products are causing the mucosal damage). The hypothesis has not, however, been tested by co-culture of the biopsies with peripheral blood lymphoid cells which, in treated coeliacs, is where the evidence suggests the gluten-reactive cells are to be found.

Third, all studies of cell-mediated immunity to gluten have compared coeliacs with controls without regard for HLA status. About 80% of coeliacs have HLA-B8 compared with about 20% of unselected controls (Falchuk et al. 1972). However, HLA-B8 has been associated with a generalised increase in immune responsiveness (Vladutiu & Rose, 1974) and increased humoral responses to gluten (Scott et al. 1974). More

recently, Cunningham-Rundles et al. (1978) have shown increased lymphocyte transformation with gluten in normal individuals having HLA-B8. It thus seems pertinent to study the influence of HLA-B8 on the immune response to gluten as measured by the LMI test.

Fourth, the importance of humoral immunity to gluten in pathogenesis has been questioned partly because of the occurrence of other dietary antibodies in coeliac disease. Tests of cell mediated immunity to dietary antigens have not been widely performed, and so far as the LMI test is concerned the only antigen tested has been α -lactalbumin (Haeney & Asquith, 1978). The specificity of the response to gluten cannot thus be properly assessed.

Finally, there is the problem of the interpretation of LMI test results. The justification for in vitro tests of cell mediated immunity essentially lies with the correlation between the results of the test and in vivo evidence of cell mediated immunity, usually a skin test. In coeliac disease skin tests have not supported the concept of cell mediated immunity to gluten and there are many alternative mechanisms by which inhibition of leucocyte migration may occur other than by T-lymphocyte produced lymphokine. These include migration inhibitors

produced by B cells (Rocklin et al. 1974) or other cell types (Tubergen et al. 1972), cytophilic antibody (Heise et al. 1968; Kostiala & Kosunen, 1972; Lockshin et al. 1973; Amos et al. 1967; Wasserman & Packalen, 1965; Ortiz-Ortiz et al. 1974), immune complexes (Spitler et al. 1967; Kotkes & Pick, 1975) and direct toxicity of antigen (Maini et al. 1973). Further investigation of the mechanism and cellular basis of the LMI test in coeliac disease is needed to justify the general assumption that it is a measure of T-cell reactivity to gluten.

III. The lectin theory

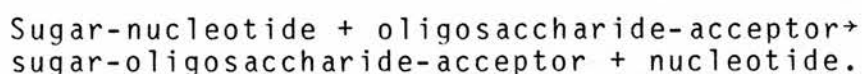
The third and most recently proposed possible mechanism for gluten toxicity is the lectin theory of Weiser & Douglas (1976). Essentially, Weiser & Douglas propose that in coeliac disease there is some abnormality of the enterocyte cell membrane which allows gluten to bind to the cells in a lectin-like manner, and that this binding is toxic to the cells. The proposed membrane abnormality is a change in the carbohydrate component of the surface membrane glycoproteins. In order to assess this theory, it is necessary to discuss some of the properties of lectins and the structure, metabolism and function of cell surface glycoproteins.

Lectins Lectins are naturally occurring proteins which have the ability to agglutinate cell suspensions (for review see Sharon & Lis, 1972). They are particularly common in plant seeds, especially legumes, but are also found in some invertebrate and lower vertebrate animals. Lectins have been used in some classical experimental work in medicine. For example, Ehrlich used the lectins ricin and abrin in the 1890s in an early demonstration of immunological specificity. In the early years of this century Landsteiner began work on the agglutination of red blood cells by lectins which has led to a much fuller understanding of human blood group systems. The observation by Nowell in 1960 that certain plant lectins stimulated lymphocytes to transform has led to an enormous amount of work on lymphocytes, their subclasses and their role in cellular immunity, and to the study of human chromosomes in vitro. Currently the observation that some lectins differentially agglutinate normal cells and cells transformed by oncogenic viruses or carcinogens has led to much work on the surface properties of malignant cells.

The mechanism of interaction between lectins and cell membranes involves specific binding of cell-surface carbohydrates and can be inhibited by simple

sugars. Most lectins interact with only a single sugar but in some the specificity is broader and includes closely related sugars (Weir, 1980). The specificity of this reaction has been compared with that of the antigen-antibody reaction.

Cell-surface carbohydrates There is considerable evidence that cell surfaces are characteristically covered in a complex array of carbohydrate moieties, often associated with proteins or lipids. Blood group and histo-compatibility antigens are two obvious examples. The biosynthesis of cell-surface glycoproteins depends on enzymes known as glycosyltransferases and is not directly dependent on RNA. Each glycosyltransferase catalyses the reaction:



Elongation of the oligosaccharide chains is effected by the addition of monosaccharide units through the action of different glycosyltransferases in a specific sequence. The product of each reaction serves as the specific substrate for the next enzyme in the sequence. The sequence of reactions does not, however, always proceed to completion, leading to micro-heterogeneity of surface glycoproteins (Roseman, 1970).

The functions of cell surface carbohydrates are not entirely understood, but it seems likely that they are intimately involved in cellular recognition and intercellular adhesion (Roseman, 1970; Roth et al. 1971; Roth & White, 1972; Roth, 1973). As informational molecules carbohydrates have certain advantages over proteins or nucleic acids due to their ability to exist in a number of spatial configurations, to be linked in a number of different ways and their ability to form branched structures. It has been calculated, for example, that a twelve residue oligosaccharide of three mannose, three N-acetylglucosamine, three galactose and three sialic acid residues has 10^{24} possible structures (Weir, 1980). There is good evidence that carbohydrates are important in cellular recognition in primitive creatures such as the slime mold (Weir, 1980) and there is also evidence in mammals from experiments with embryo neural retina cells (Roth et al. 1971) and platelets (Chesney et al. 1972). The binding of bacteria to macrophages also seems to be mediated via a lectin-like attachment (Weir & Ogmundsdottir, 1977). Interestingly in this last case the lectin-like molecule seems to form part of the phagocyte cell wall.

The theory itself Weiser & Douglas (1976) point out that the number of incomplete carbohydrate chains is increased in virally transformed cells, intestinal tumour cells and normal intestinal crypt cells (Burger, 1973; Podolsky & Weiser, 1973). Immature intestinal epithelial cells exhibit different lectin-binding patterns to mature enterocytes and have altered glycosyltransferase activities and endogenous acceptors (Etzler & Branstrator, 1974; Weiser, 1973a, 1973b). Transformed intestinal cells are more susceptible to lectin-induced cytotoxicity in vitro than normal cells (Shoham et al. 1970). It is suggested that in coeliac disease a defect in cell-membrane carbohydrates allows gluten (or a fraction thereof) to bind in a lectin-like manner to the incomplete carbohydrate and that this binding is toxic to the cell. Transient gluten intolerance is explained by the appearance on the villus of immature epithelial cells as the epithelium attempts to repair itself after an insult such as viral enteritis.

The theory is an attractive one, especially considering the evidence for carbohydrate involvement in intercellular adhesion as one can easily envisage a lectin interfering with this process so that cells are more easily shed. Increased enterocyte shedding is a

feature of untreated coeliac disease (Croft et al. 1968). However, the evidence above is all circumstantial, and direct experimental evidence is scanty.

Experimental evidence relating to the lectin theory

The most basic piece of evidence to support the lectin theory would be the demonstration that toxic gluten peptides bind to coeliac jejunal mucosa but not to normal mucosa. Perhaps surprisingly there is little evidence that this is so. Rubin et al. (1965) used an immunofluorescent technique and showed binding of gliadin to the jejunal mucosa of nine adult coeliacs. Binding seemed maximal in the crypt cells and fluorescence was mainly cytoplasmic. Only one of five controls showed any positive fluorescence. This work, however, has never satisfactorily been repeated. Hobbs (1974) comments that his preparation of rhodamine-labelled gliadin tended to "stick to everything" and could not be used for localisation studies. In fact, the only other positive report in this area comes from Douglas himself (Douglas, 1976). He prepared a glycoprotein extract of gluten (termed glyc-gli) by acetic acid-ethanol extraction followed by trichloroacetic acid precipitation and labelled this with ^{125}I . The

glyc-gli was shown to be toxic in vivo. When incubated with intestinal homogenates binding could be shown to mucosa from treated and untreated coeliacs, but only minimally to mucosa from controls. Iodinated α -gliadin did not bind to any specimens. The binding of glyc-gli was reduced by 40% by incorporation of five millimolar rhamnose and 30% by arabinose, suggesting a sugar specificity. Oddly, galactosamine increased binding to coeliac mucosa by 46%. With normal mucosa binding was increased by galactosamine and arabinose but unaffected by rhamnose, which thus seemed to be the sugar specificity of the putative lectin.

Weiser & Douglas (1978) have also studied the glycosyltransferase activities of isolated enterocytes in coeliac disease. They found an increased activity of galactosyltransferase in coeliacs compared with controls with both endogenous and exogenous acceptors. With endogenous acceptors (those naturally occurring on the cell surface) the kinetics of the reaction with this enzyme were also altered, suggesting an increase in the number of endogenous acceptor sites. Results did not correlate with histological appearance or time on a gluten free diet, suggesting that the changes were not merely secondary to mucosal damage and the presence of immature cells on the villi. However, the methods

used are open to criticism as they are technically very difficult, were performed on relatively few patients and, for technical reasons, factors such as cell "leakiness" and cell surface hydrolytic enzymes were not evaluated.

Machell (1979) has studied the binding of a range of fluorescein-labelled lectins of known sugar specificities to enterocytes from coeliac patients and normals, using the lectins as probes to identify carbohydrate moieties on the cell membranes. He found no differences between coeliac and control biopsies.

Finally, it is worth noting that the previously discussed work of the Galway group (Stevens et al. 1978) concerning the role of carbohydrate in gluten toxicity may be relevant to the lectin theory.

One problem with the lectin theory is the scarcity, and technical difficulty, of experimental test systems. One possibility which has not been explored is assay of cell membrane inositol phospholipid turnover, as there are some grounds for believing that this may be affected by lectin binding to cell membranes.

Phosphatidylinositol turnover in cell membranes

Myoinositol was originally described as an essential growth factor early this century, yet its function remained unclear until recently when it was discovered

that most of the inositol in eukaryote cells exists in combined form in the phospholipid of cell membranes. The metabolism of these inositol phospholipids is now well worked out (see review by Michell, 1975). The metabolism is in the form of a cycle (shown in Fig.2). Phosphatidylinositol is synthesised from 1,2, diacylglycerol via phosphatidic acid. In response to various stimuli it is broken down to release the 1,2, diacylglycerol backbone which is then reutilised in the re-synthesis of phosphatidylinositol. The rate of degradation of the phosphatidylinositol (hereafter referred to as the PI turnover) can thus be measured in two ways: (1) by measuring the incorporation of labelled inorganic phosphate into phosphatidylinositol (which necessitates separation of the cell membrane lipids as phosphate is also incorporated into other phospholipids) or (2) by use of labelled inositol which is specifically incorporated into phosphatidylinositol. The importance of the PI turnover cycle lies in its relation to the interaction of cell membranes with a variety of stimuli. Increased PI turnover has been described in a wide variety of tissues in response to a wide variety of stimuli (see Table 3). It is noteworthy that the stimuli share the common feature that they exert their effects by interaction with cell

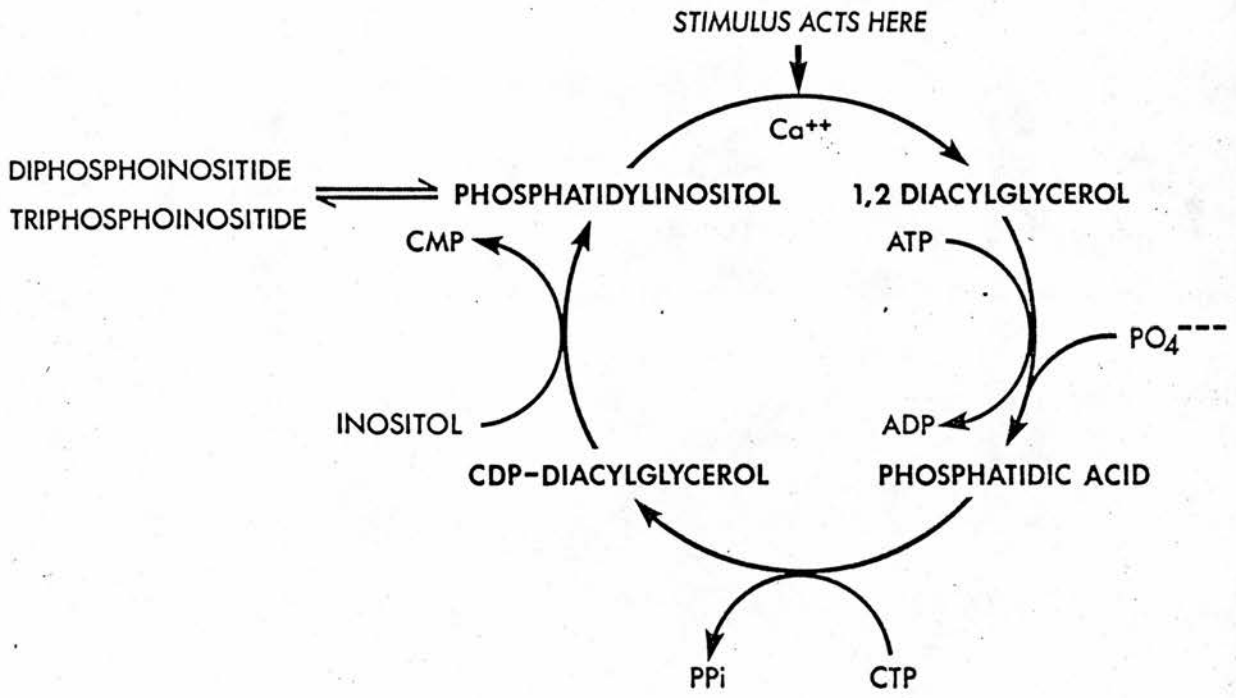


FIG. 2. The phosphatidylinositol turnover cycle

TABLE 3

Stimuli reported to increase phosphatidylinositol turnover and the tissues affected

<u>Stimulus</u>	<u>Tissue(s) affected</u>
Cholinergic	Cerebral cortex (and other brain regions) Sympathetic ganglia Synaptosomes (and subcellular fractions containing them) Pineal gland Adrenal medulla Pancreas Parotid gland Submaxillary gland Avian salt gland Electrogenic tissue Thyroid gland Peptic mucosa Sweat glands
Adrenergic	Cerebral cortex (and other brain regions) Synaptosomes Pineal gland Parotid gland Submaxillary gland Heart Liver Platelets Adipose tissue Vas deferens smooth muscle
Histamine	Gastric mucosa
5-Hydroxytryptamine (serotonin)	Cerebral cortex (and other brain regions) Pineal gland
Dopamine	Cerebral cortex (and other brain regions)
ADP	Platelets
Glucose	Islets of Langerhans
Pancreozymin/cholecystokinin	Pancreas
Caerulein	Pancreas
Thyroid-stimulating hormone	Thyroid gland
Long-acting thyroid stimulator	Thyroid gland
Insulin	Adipose tissue
Corticotrophin-releasing hormone	Anterior pituitary
Thrombin	Platelets

-ctd.

Table 3 ctd.

<u>Stimulus</u>	<u>Tissue(s) affected</u>
Collagen	Platelets
Phytohaemagglutinin	Lymphocytes (T?)
Lens culinaris lectin	Lymphocytes (T?)
Concanavalin A	Lymphocytes (T?)
Soya bean lectin	Lymphocytes (T?)
Wistaria floribunda lectin	Lymphocytes (T?)
Antilymphocyte antiserum	Lymphocytes (B & T?)
Anti-immunoglobulin antiserum	Lymphocytes (B?)
Serum components or viral transformation	Fibroblasts
Particles (latex, starch)	Polymorphonuclear leucocytes Macrophages
Bacterial endotoxin	Polymorphonuclear leucocytes Macrophages
Stapylococcal leucocidin	Polymorphonuclear leucocytes
Digitonin	Polymorphonuclear leucocytes Macrophages
Deoxycholate	Polymorphonuclear leucocytes Macrophages
Electrical (parasympathetic) (sympathetic) (sympathetic pre-ganglionic) either in situ or in vitro (field stimulation)	Submaxillary gland Submaxillary gland Sympathetic ganglia Cerebral cortex Synaptosomes
High K ⁺ (depolarising)	Cerebral cortex Sympathetic ganglia Synaptosomes Electrogenic tissue
Flight (= glutamate?)	Cockroach flight muscle

(from Michell, 1975)

surface receptors. It is also noteworthy that the stimuli include several lectins. Studies involving lectins date back to 1968 when Fisher & Mueller reported increased PI turnover in lymphocytes in response to phytohaemagglutinin. Later work (Mario et al. 1975) showed that other lectins had the same effect but only if they stimulated lymphocyte transformation. Non-mitogenic lymphocytes had no effect.

What relevance does this have to coeliac disease? If, as is postulated by Weiser & Douglas, gluten affects coeliac enterocytes by a lectin-like binding to the cell membrane, then it is at least possible that a marker for this reaction may be an increase in PI turnover in these cells. With the advent of jejunal mucosal biopsy organ culture it is now technically feasible to study phospholipid turnover in jejunal mucosa and the system has been used to study lipoprotein synthesis and secretion (Rachmilewitz et al. 1980), but PI turnover and the effects of gluten have not been studied.

The lectin theory and the LMI test

One final point to consider is the possibility that some of the experimental results discussed as relevant to the immunological theory may be explained by the lectin theory.

Many lectins are mitogenic for lymphocytes and, consequent on this non-specific activation, cause the release of lymphokines. The lectin from the asparagus pea has also been shown to elicit a direct chemotactic effect from human polymorphs (Van Epps & Tung, 1977). It is thus possible that some of the results of transformation assays and the LMI test may be explained by a lectin effect of gluten rather than in terms of an immune response to gluten. If this were so the effects could be blocked by the sugar specific for the lectin - in the case of gluten Douglas' results (Douglas, 1976) suggest that this is rhamnose. This possibility has not been experimentally explored.

AIMS OF THE STUDY

- 1) To further evaluate the direct Leucocyte Migration Inhibition test in coeliac disease with particular reference to:
 - (a) the influence of histocompatibility type on results of the test in normal controls and in coeliac patients
 - (b) the effect of dietary status on results
 - (c) the specificity of the test with respect to the antigen used; in particular, to determine whether positive results are obtained in coeliac disease with dietary antigens other than gluten
 - (d) the relationship between production of inhibitory factors by jejunal mucosa and by peripheral blood leucocytes
 - (e) a comparison of two subfractions of gluten as antigen
 - (f) assessment of whether gluten may be affecting results by means of a direct lectin-like binding to leucocytes

- (g) an investigation into the mechanism by which gluten causes migration inhibition and into the assumption that it does so by inducing lymphokine production by specifically sensitised T lymphocytes
- 2) To extend the use of jejunal mucosal biopsy organ culture techniques in investigating pathogenesis:
- (a) by co-culturing biopsies from treated coeliacs with their own peripheral blood lymphocytes in an attempt to restore gluten sensitivity to these biopsies
 - (b) by using the technique to measure phospholipid turnover in the jejunal mucosa as a possible index of a lectin-like action of gluten

M A T E R I A L S & M E T H O D S

"Let us work without theorizing.....'tis the only way
to make life endurable"

Candide
Voltaire, 1759.

Patients

As discussed in the Introduction, all coeliac patients participating in these studies fulfilled one of the following criteria for the diagnosis of coeliac disease:

- 1) a jejunal biopsy taken whilst the patient was on a normal diet which showed typical changes of coeliac disease, with a second biopsy taken after a period of gluten exclusion which showed unequivocal histological improvement or:
- 2) an initial biopsy taken whilst the patient was on a gluten-free diet with a second biopsy taken after a gluten challenge with clear histological deterioration in the second biopsy compared with the first.

A total of 58 patients was studied. Twenty eight were male and 30 female. Mean age was 36.8 years (range 11 - 68 years).

Normal controls. A total of 31 normal healthy controls, drawn from hospital personnel was studied. There were 16 males and 15 females. Mean age was 33.5 years (range 20 - 54 years).

Biopsied controls. A group of 20 patients undergoing routine jejunal biopsy for the exclusion of coeliac

disease was also studied. These comprised eight men and 12 women, mean age 29.7 years (range 16 - 56 years). Jejunal biopsies showed no evidence of coeliac disease in any case. Final diagnoses were as follows:

Irritable Bowel Syndrome	-	6
Crohn's disease	-	3
Family history of coeliac disease	-	3
Anaemia ? cause	-	3
Chronic pancreatitis	-	1
Anorexia nervosa	-	1
Aphthous ulceration	-	1
Abdominal lymphoma	-	1
Food allergy	-	1

Details of the patients and controls used in each experiment are given in the appropriate section.

The Leucocyte Migration Inhibition Test

General technique. The LMI test was performed routinely using a capillary tube method as described by Bullen & Losowsky (1978). 20ml of venous blood was withdrawn under sterile conditions and defibrinated by repeated inversion in a 30ml sterile plastic Universal container (Sterilin, L.I.P. Ltd., Shipley) containing 8-10 sterile glass beads. The fluid defibrinated blood was then decanted into another Universal container and

mixed with 10ml of Dextran 150 in saline (Dextraven 150, Fisons Ltd.). This was then allowed to sediment for 30 minutes at room temperature and the leucocyte-rich supernate aspirated using a sterile 10ml syringe. This cell suspension was then centrifuged at 200 x g for 10 minutes to pellet the cells and the supernate discarded. The cells were then washed twice in medium and finally adjusted to give a packed cell volume of about 50%. This gave a leucocyte concentration of about 10⁷ cells/ml. The medium used was Medium 199 (Wellcome Reagents) containing 10% foetal calf serum (FCS) (Wellcome Reagents), 1000 units/ml each of penicillin and streptomycin (prepared by the Pharmacy, St. James's University Hospital, Leeds) and adjusted to pH 7.4 with 8.4% sodium bicarbonate (Pharmacy Dept., St. James's University Hospital).

The cell suspension was then drawn up into 10 μ l micro-capillary tubes. These were Drummond Microcaps (20 μ l) (L.I.P. Ltd.) which had been scored with a diamond and broken into halves, then resterilised with dry heat (160^oC for one hour). The cell suspension was then drawn up to leave a slight gap at one end of the tube and this end was heat sealed in a butane gas flame. Care was taken not to char the cells. The filled capillaries were transferred to another sterile Universal and the cells gently packed by centrifugation at 50 x g for three minutes. The tubes were then

scored with a diamond and broken at or just below the cell-fluid interface before being placed in the Leucocyte Migration plates. Tubes showing evidence of cell charring, haemolysis or fragmentation of the cell column were discarded. The Leucocyte Migration plates (Sterilin Ltd.) had the rims of their wells coated with sterile silicone grease. The sealed end of the capillary tube was also dipped into silicone grease to fix its position in the well. The wells were then filled with the appropriate medium and sealed with Chance Proper cover slips (22x22mm, No 1½; L.I.P. Ltd.) taking care to exclude air bubbles and the plates incubated at 37°C. The total time between filling of the capillary tubes to placing the plate in the incubator was never more than 30 minutes, and the cut ends of the filled capillaries were never exposed to the air for more than 30 seconds. These details are critical and have been shown to affect the result of the test (Maini et al. 1973). All manipulations were performed in a Pathfinder sterile cabinet (Pathfinder, Havant, Hants.).

All cultures were performed in quadruplicate. After incubation at 37°C (standard incubation time was 20 hours - see below) the plates were read by projection of the fans of migrating cells at constant magnification using an overhead projector. The

projected images were traced onto paper and their areas measured with a fixed arm planimeter (Shandon Scientific Co. Ltd.). Any wells showing evidence of infection (this occurred on only one occasion), pH change or air bubbles were discarded.

Results are expressed as a Migration Index which is calculated as:

Migration
Index=

$$\frac{\text{Mean area of migration in the presence of antigen}}{\text{Mean area of migration in control medium}}$$

Thus a lower Migration Index indicates increased immunity.

Antigens

Gluten fraction III was made from BDH wheat gluten by the method of Frazer et al. (1959) and was freeze-dried.

Gluten subfraction B2. 10mg of B2 was kindly supplied by Dr. Azaria Ashkenazi, Kaplan Hospital, Rehovot, Israel.

Purified Protein Derivative (PPD). Lyophilized preservative-free PPD was obtained from the Ministry of Agriculture, Fisheries and Food, Weybridge.

Dietary antigens. The dietary antigens studied were prepared as described below. Relatively crude antigens were used deliberately, for two reasons: (1) similar crude preparations have been used in most reported antibody studies in coeliac disease and (2) it seemed more likely that crude extracts of foods would contain antigens actually found in the diet than would the highly purified food extracts which are commercially available.

Egg. 15g of hard-boiled egg white was homogenised in 15ml of 0.9% saline, filtered and sterilised by autoclaving. This stock solution was then diluted in medium to the appropriate concentration. The protein content of the stock solution (estimated by the method of Lowry et al. 1951) was 5mg/ml.

Milk. Fresh pasteurised cow's milk was centrifuged (250xg, 10 minutes) to separate the bulk of the fat and the aqueous layer diluted with medium to the appropriate concentration. Protein content of the aqueous layer was 35mg/ml (Lowry et al. 1951). No problems with infection of cultures were found, despite there being no sterilisation of the milk.

Bovine Serum Albumin (BSA). Lyophilised BSA (Sigma Ltd.) was dissolved directly in culture medium without sterilisation.

Antigen Concentration

Final concentration of antigen was determined in the case of gluten fraction III, milk, egg and BSA by performing dose response studies in controls and coeliacs. For gluten fraction III four controls and four coeliacs were studied with final antigen concentrations of between $200\mu\text{g/ml}$ and 4mg/ml . Incubation time for the dose response studies was 20 hours. Both the leucocyte migration index and post-culture leucocyte viability (assessed by trypan blue exclusion (Boyse et al. 1964)) were used to determine the appropriate concentration. Results of the migration index study are given in Figure 3 and of the viability data in Figure 4. At low antigen concentrations ($200\mu\text{g/ml}$ and $500\mu\text{g/ml}$) there is little evidence of migration inhibition. At 1mg/ml and 2mg/ml there is inhibition of migration, especially of coeliac leucocytes, though 2mg/ml also produces some inhibition of normal cells. 4mg/ml inhibits both normal and coeliac leucocytes and this is reflected in some loss of viability (Fig. 4). A final concentration of 1mg/ml was thus chosen. This is in accord with previous work (Bullen & Losowsky, 1978).

For egg, milk and BSA similar studies were undertaken in three coeliacs and three controls, with

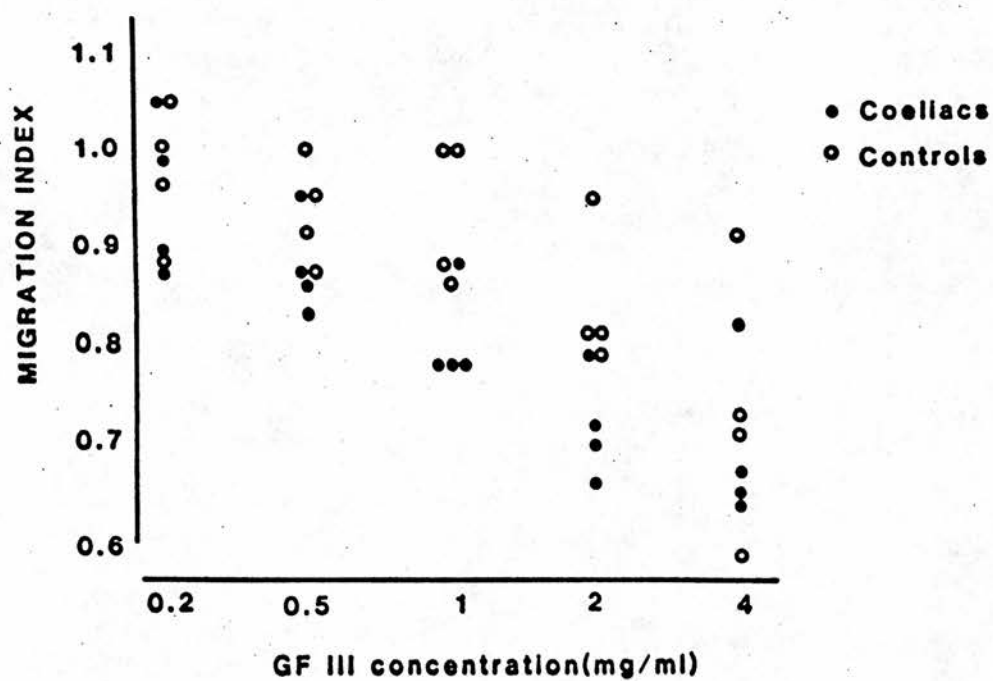


FIG. 3. Dose response in LMI test for gluten fraction III - migration indices

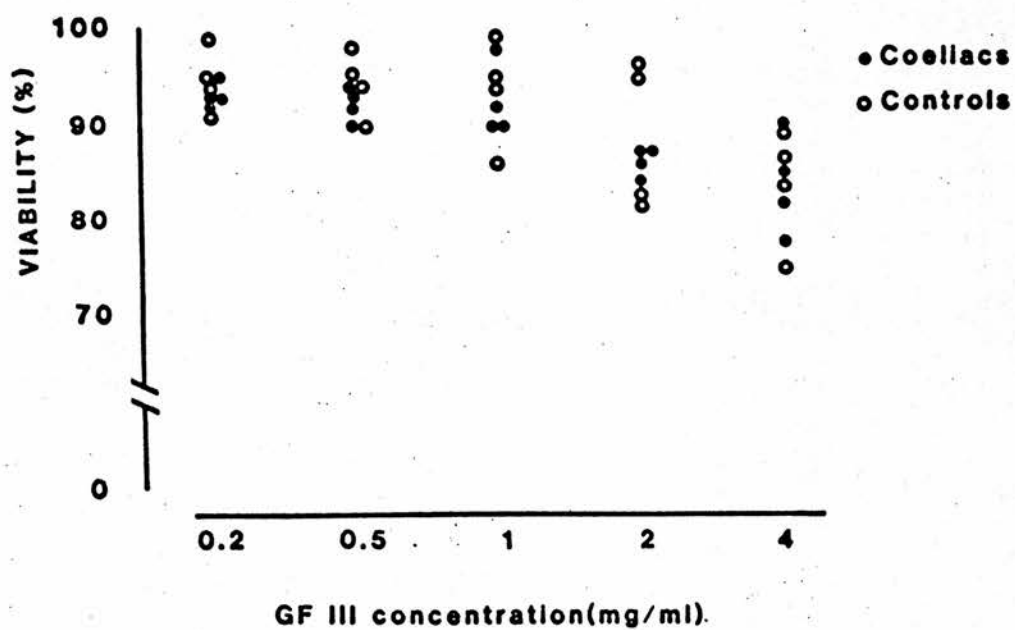


FIG. 4. Dose response in LMI test using gluten fraction III - leucocyte viabilities after 20 hrs. culture

final antigen concentrations of 1, 2 and 4mg/ml of BSA, 1%, 2% and 4% (v/v) of the stock egg white antigen solution and 1%, 2% and 4% (v/v) of centrifuged milk. Results for migration indices are given in Figure 5. For BSA and egg the higher antigen concentrations caused some inhibition of normal cells and final concentrations of 1mg/ml and 1% (v/v) of stock solution were chosen. In the case of milk there was little difference between 1% and 2%. 1% was chosen largely because the plates were more easily read as the culture medium was less cloudy. Mean leucocyte viabilities with the chosen final antigen concentrations were 91% for egg, 93% for BSA and 93% for milk with normal leucocytes and 92% for egg, 91% for BSA and 91% for milk using coeliac leucocytes. Results at higher antigen concentrations were similarly close, showing no increased susceptibility of coeliac leucocytes to toxicity of these antigens compared with controls.

PPD was used at a concentration of 200 μ g/ml (most published studies use 100 - 300 μ g/ml). B2 was used at 10 μ g/ml to allow comparison with published results (Ashkenazi et al. 1978). As only 10mg of this material was available, higher concentrations were impracticable.

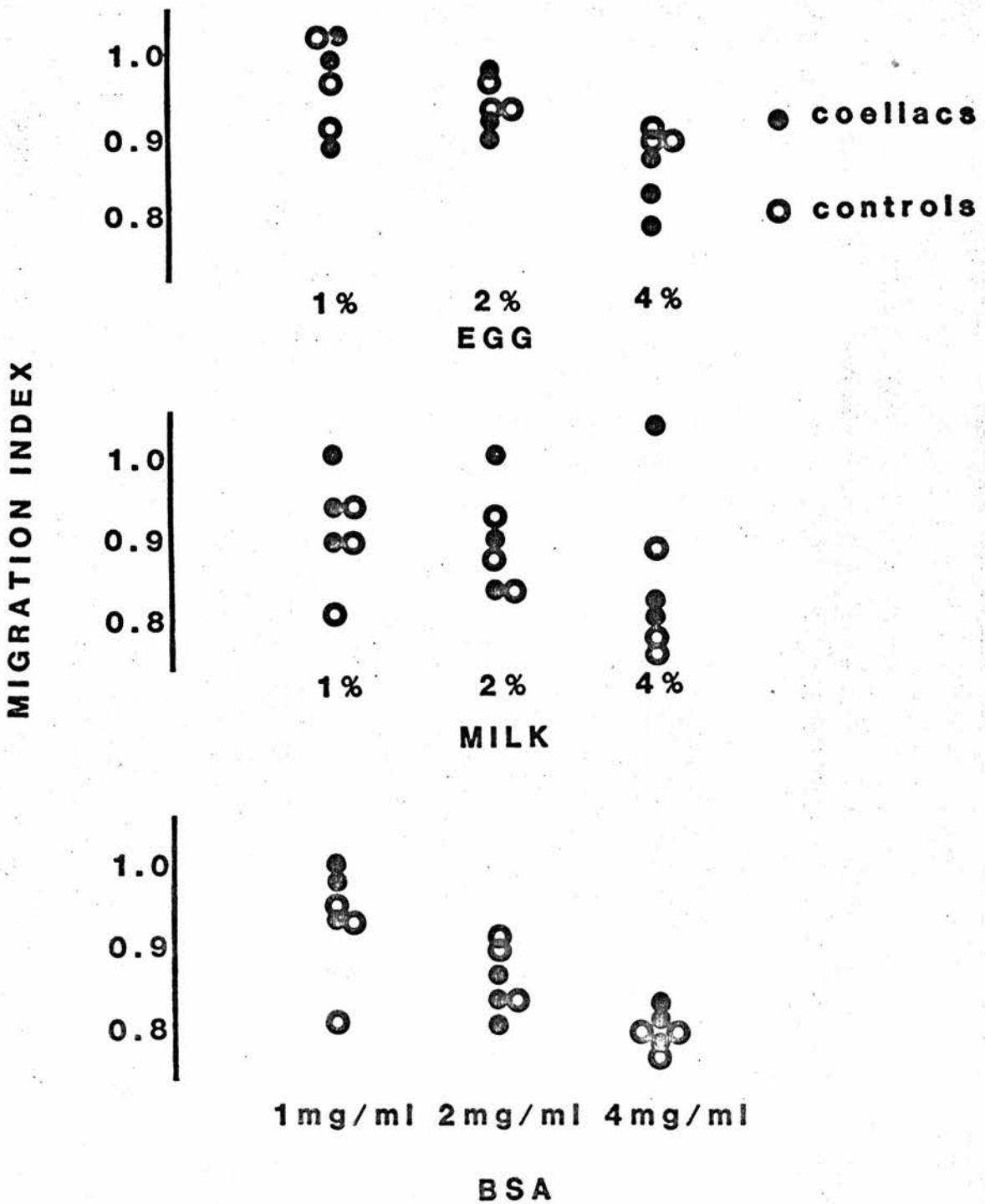


FIG. 5. Dose response in LMI test with the dietary antigens egg white, milk and BSA

Duration of incubation. The optimal duration of incubation was determined using gluten fraction III at 1mg/ml as antigen in four normal controls and four coeliacs by measuring migration indices at 5-hour intervals. Results are shown in Figure 6. Some inhibition of migration of coeliac leucocytes is seen as early as five hours but maximal separation of coeliacs from controls occurs at 15 hours and 20 hours. Twenty hours was chosen as the standard incubation time as it proved more convenient in practice.

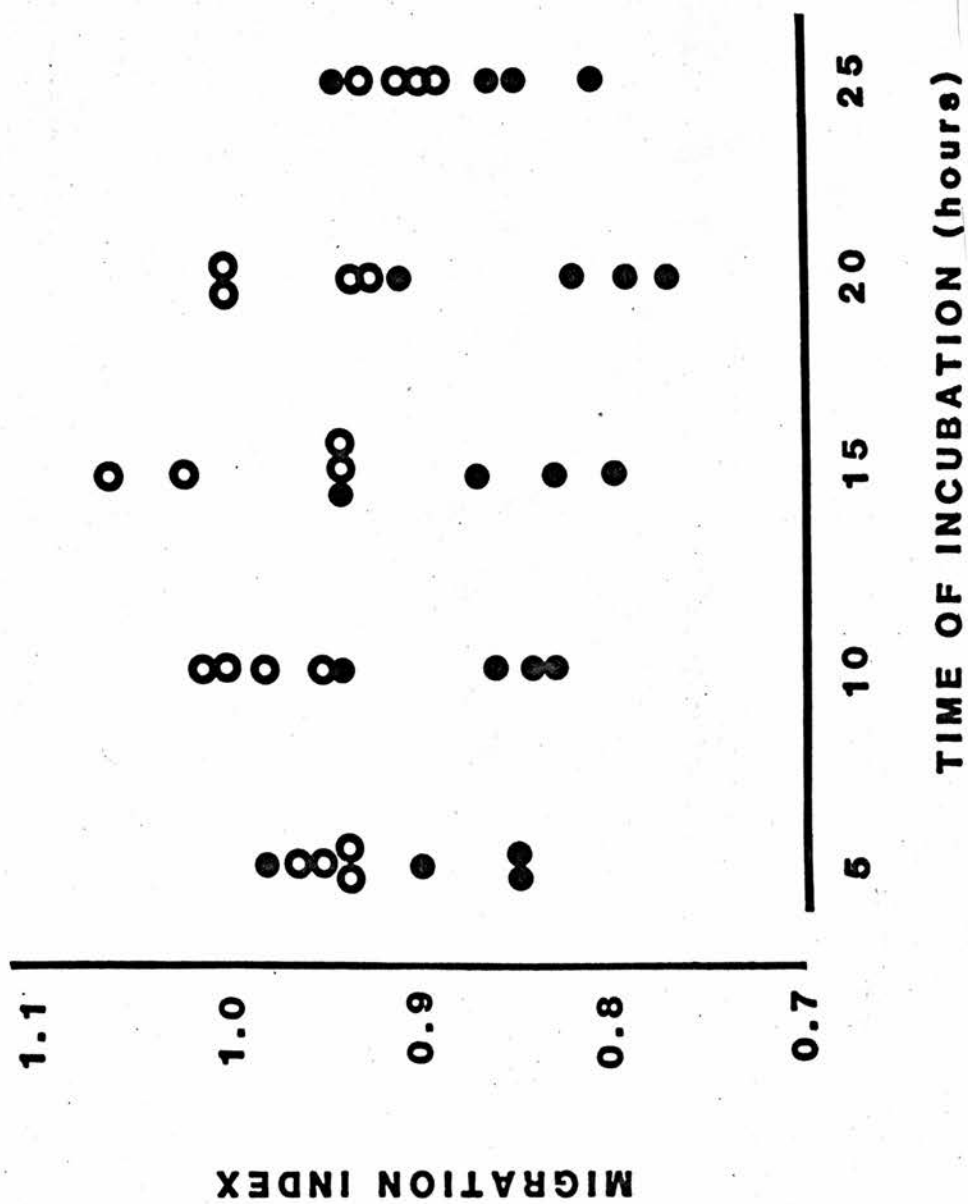
Reproducibility of LMI test

This was assessed in several ways. First, 14 individuals (9 controls and 5 coeliacs) had their migration indices to gluten fraction III determined on two separate occasions (without any alteration in dietary status). The correlation between the first and second results is shown in Figure 7. The correlation is good, with a correlation coefficient $r = 0.831$, ($p < 0.01$). The same results are tabulated in Table 4. From these results the calculated SD for duplicates is 0.0354, giving a coefficient of variation of 3.84%. It can also be calculated that differences equal to or greater than $1.96 \times \sqrt{2} \times 0.0354 = 0.098$ are unlikely to be due to experimental error ($p < 0.05$) (Campbell & Owen, 1967).

FIG. 6. Time course of LMI test using gluten fraction III

● coellacs

○ controls



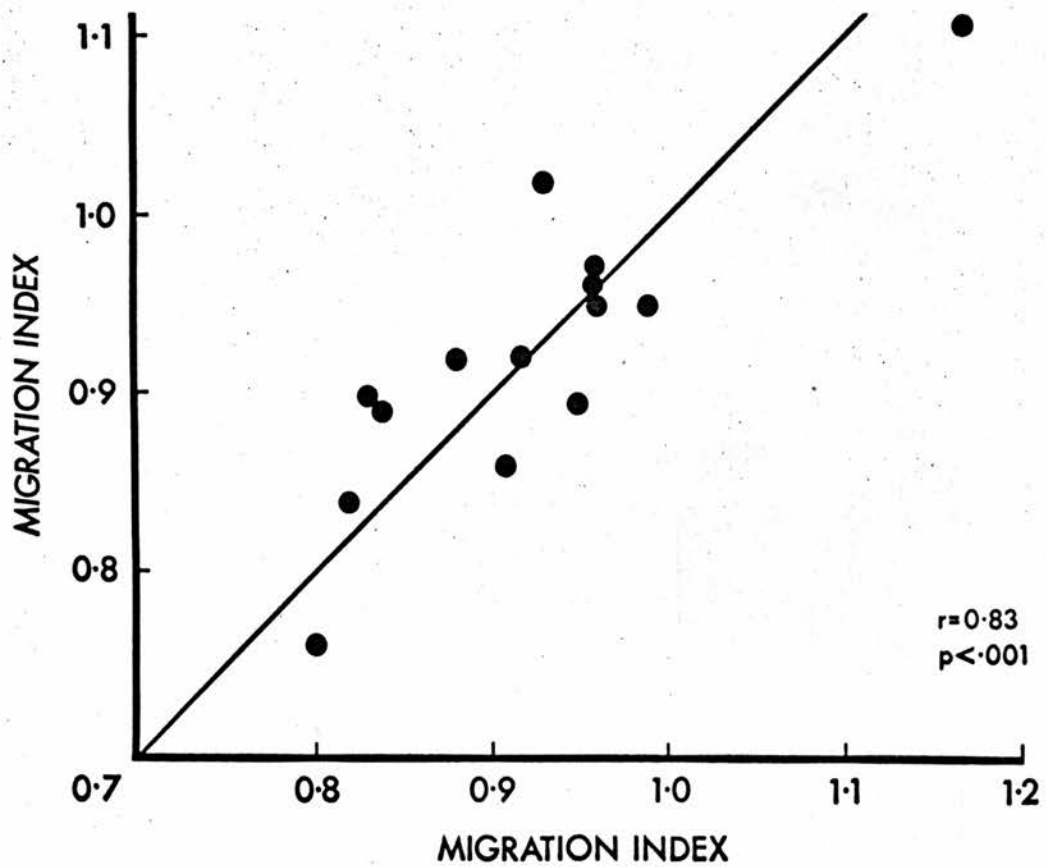


FIG. 7. Correlation between migration indices in the LMI test using gluten fraction III on 14 individuals studied on 2 separate occasions

TABLE 4

Results of migration indices to gluten fraction III in 14
individuals determined on two separate occasions

<u>1st Migration Index</u>	<u>2nd Migration Index</u>	<u>Difference</u>
0.84	0.82	+ .02
0.95	0.96	- .01
0.89	0.95	- .06
1.08	1.17	- .09
0.92	0.92	0
0.86	0.91	- .05
0.76	0.80	- .04
0.95	0.99	- .04
0.92	0.88	+ .04
0.96	0.96	0
0.89	0.84	+ .05
1.02	0.93	+ .09
0.97	0.96	+ .01
0.90	0.83	+ .07

$$\text{SD for duplicates} = \sqrt{\frac{\sum d^2}{n}} = 0.0354$$

$$\text{Coefficient of variation} = \frac{\text{SD}}{\text{overall mean}} = \frac{0.0354 \times 100\%}{0.9225} = 3.84\%$$

Second, one normal control had his migration index to gluten measured on six separate occasions. Results are given in Table 5 and give a coefficient of variation of 9%.

Finally, the coefficient of variation of the migration areas of the quadruplicate wells was calculated for 25 consecutively studied individuals (12 controls, 13 coeliacs) both for control cultures and for those with gluten fraction III. The mean coefficient of variation overall was 8.5% (range 0.1 - 16.4%). There was no difference between variability in coeliacs versus controls nor for control medium versus medium with added gluten.

Influence of HLA type and diet on LMI test using gluten fraction III

Subjects. Fifty eight patients with coeliac disease and 30 healthy controls were included in this study. Twenty six of the patients were studied whilst taking a normal diet. Coeliacs studied whilst on a gluten-free diet were arbitrarily divided into an early treatment group (gluten-free diet (GFD) for between 3 and 12 months) and a late treatment group (GFD for over one year). Twenty five patients were studied whilst in the early treated category (mean duration of GFD 6.2 months, range 3 - 11 months) and 32 whilst in the late

TABLE 5

Migration index to gluten fraction III in a normal control measured on six separate occasions

Migration Index

0.89)
0.95)
0.98)
0.77)
0.93)
1.00)

Coefficient of Variation = 9%

treatment category (mean duration of GFD 5.4 years, range 15 months - 10 years). Six patients were studied serially at all stages of treatment.

HLA typing. Typing for A and B loci was performed by the standard National Institute of Health, Bethesda technique which is based on the microlymphocytotoxicity technique of Terasaki & McLelland (1964). A bank of 60 sera was used. Typing was performed by the Regional Blood Transfusion Centre, Leeds. All patients and controls were typed for A and B loci.

DR typing was performed on 20 normal controls. This was done by a modification of the 2-colour fluorescence method of Van Rood et al. (1976) and was performed by the University Department of Medicine at the Royal Liverpool Hospital.

LMI test with gluten and other dietary antigens

LMI testing. LMI tests using gluten fraction III and the dietary antigens egg white, milk and BSA (at the concentrations described above) were simultaneously performed on 28 coeliac patients (15 men and 13 women). Mean age was 36 years (range 12 - 60 years). Ten patients were studied untreated; 10 were studied early in treatment (mean duration of GFD 5.6 months, range 4 - 8 months) including nine patients who had been studied untreated; the remaining 16 patients were

in the late treatment group (mean duration of GFD 5.3 years, range 15 months - 10 years). Twenty four healthy controls of similar age and sex distribution were also studied.

Jejunal morphology. Jejunal biopsies were obtained from the region of the ligament of Treitz using the Quinton capsule within 24 hours of performing the LMI test in all 10 untreated coeliacs, eight of the early treated group and 11 of the late treated group. The biopsies were graded both by stereomicroscopic and histological appearance (Scott, 1975) to allow changes in immune responses to the dietary antigens to be related to the jejunal villous morphology.

Biopsied controls. All 20 biopsied controls were studied with all four dietary antigens. All were also HLA typed for A and B loci.

Leucocyte inhibitory factor production by jejunal mucosa

The relationship between production of leucocyte inhibitory factors by jejunal mucosa and by peripheral blood lymphocytes was investigated in 16 coeliac patients (8 men, 8 women) by the simultaneous performance of a direct LMI test with gluten fraction III on blood leucocytes and an indirect LMI test using cultured jejunal biopsies. Eight patients were studied

whilst untreated and nine after treatment (mean duration of GFD 2.7 years, range 8 months - 7 years). One patient was studied untreated and after eight months of treatment. The direct LMI test was performed as described above.

Indirect LMI test. Jejunal biopsies from the region of the ligament of Treitz were placed into organ culture (details of the organ culture technique are given later) with and without the addition of 1mg/ml gluten fraction III to the culture medium. After five hours culture the medium was removed and stored at -20°C until the indirect LMI test was performed. This was done using leucocytes from a single healthy control. The test was performed in the same way as the direct LMI test but the culture medium used was that in which the jejunal biopsies had been cultured. To negate any non-specific action of gluten the control medium was reconstituted with 1mg/ml of gluten fraction III. The only difference between the test and control media was thus the presence of any factors produced by the jejunal biopsy in response to being exposed to gluten in culture. Migration index was thus calculated as:

$$\text{Migration Index} = \frac{\text{Mean area of migration in test medium (from biopsies cultured with gluten)}}{\text{Mean area of migration in control medium (gluten added after culture)}}$$

Comparison of B2 and gluten fraction III

A direct comparison of subfraction B2 and gluten fraction III in the direct LMI test was made in 15 individuals (6 normal controls, 5 untreated coeliacs and 4 coeliacs on treatment with gluten-free diet for between 3 and 12 months). The concentration of B2 used was that reported to be optimal in the agar drop system, i.e. 10µg/ml (Ashkenazi et al. 1978; 1980). Gluten fraction III was used at 1mg/ml.

Effect of rhamnose on LMI test with gluten

To assess whether gluten might be inhibiting the migration of coeliac leucocytes by a lectin-like binding to leucocyte cell membranes, six coeliac patients (1 untreated, 5 on a gluten-free diet for a mean of 5 months) were studied using the LMI test with gluten fraction III with and without the addition of the monosaccharide rhamnose (Sigma Chemicals Ltd.) at 5mmol/l. This concentration of rhamnose was chosen as it was reported by Douglas (1976) to inhibit attachment

of his gluten subfraction glyc-gli to cell membranes. The effect of rhamnose alone at this concentration on the migration of leucocytes was also investigated in the same six patients.

Effect of puromycin on LMI test

Puromycin and other protein synthesis inhibitors have been shown to block lymphokine-mediated inhibition (Mitchell et al. 1972; Maini et al. 1973; Gorski, 1974). Its effect on results of the LMI test with gluten were thus investigated. The method was first validated using PPD - an antigen for which the importance of T cell-mediated responses is much better documented.

Puromycin and the LMI test with PPD

Five normal controls were Mantoux-tested with 1 I.U. of PPD (St. James's University Hospital Pharmacy), injected intradermally. The diameter of induration produced was measured at 24 hours. Induration of greater than 10mm was taken as a positive response. The LMI test was performed using cells from these five individuals with preservative-free PPD (Weybridge) at a final concentration of 200 μ g/ml, with and without the addition of puromycin (St. James's University Hospital Pharmacy) at a final concentration of 15 μ g/ml. This concentration of puromycin has been shown to be effective in blocking lymphokine action (Mitchell et al. 1972; Maini et al. 1973).

Puromycin and the LMI test with gluten

The effect of puromycin at 15 μ g/ml on the LMI test with gluten fraction III as antigen was investigated in six normal controls and six coeliacs (1 untreated, 5 treated with a GFD for a mean of 8 months, range 4 - 12 months).

Puromycin at the concentration used has been reported to inhibit leucocyte migration per se (Mitchell et al. 1972). This was checked in the 12 individuals studied in this series of experiments by including a set of wells with added puromycin alone.

LMI test using sub-populations of leucocytes

This was performed to investigate the assumption that results of the LMI test with gluten represent a measure of T lymphocyte mediated immunity to gluten. This was done by separating leucocytes into purified populations of polymorphonuclear leucocytes (polymorphs) and mononuclear cells, with subdivision of the mononuclear cell population into T cell-enriched and B cell-enriched populations. The LMI test was then performed using purified polymorphs, purified mononuclear cells and polymorphs with added T cells and with added B cells. Differential white cell counts on mixed leucocytes prepared by the usual dextran sedimentation method showed a mean of 63% polymorphs,

33% lymphocytes and 4% eosinophils and monocytes. Accordingly, in the experiments where T-enriched or B-enriched lymphocytes were added to polymorphs this was done in the proportion of 2:1 polymorphs: lymphocytes in order to approximately reproduce the proportions seen with the standard LMI test.

Separation of leucocyte subpopulations

Mononuclear cells were separated from heparinised venous blood by centrifugation at 400xg for 30 minutes through Histopaque 1077 (Sigma Chemicals Ltd.) after dilution of the blood with an equal volume of sterile phosphate buffered saline of pH 7.2. The mononuclear cells were aspirated from the interface. Mean yield of mononuclear cells from 30ml of blood was 3.2×10^7 cells. Differential counts showed >95% lymphocytes with the remainder being monocytes. The red cell pellets from beneath the Histopaque were combined with minimal agitation and allowed to sediment at 37⁰C for 20 minutes. The polymorph-rich supernate was then aspirated. Mean yield of polymorphs was 3.14×10^7 cells. Leishman stained smears of these preparations showed >99% polymorphs with only occasional eosinophils and no mononuclear cells. Aliquots of the mononuclear cell preparation were taken for use in the LMI test and the remainder used for separation into T- and B-enriched populations.

T-B cell separation

This was achieved by differential centrifugation through a Ficoll-Triosill solution after rosetting of the T lymphocytes with neuraminidase treated sheep red blood cells (based on the method of Weiner et al. 1973). Five ml of 2% sheep red blood cells (SRBC) (Oxoid Ltd.) in phosphate buffered saline (PBS) were mixed with 150 μ l of 1 unit/ml neuraminidase (Behringwerke AG, Marburg) and incubated at 37⁰C for 30 minutes. The SRBC were then washed three times in PBS and stored at 4⁰C until used. Fresh neuraminidase treated SRBC were made up weekly. T-rosetting was performed by mixing 2ml of 2% neuraminidase-treated SRBC with 10⁷ mononuclear cells and centrifuging at 200xg for 5 minutes. After one hour at room temperature, the cell pellet was gently resuspended and 1.15ml Histopaque introduced carefully under the cells with a Pasteur pipette. After centrifugation at 800xg for 20 minutes the rosetted T cells were pelleted under the Histopaque and the non-rosetted cells were at the medium-Histopaque interface. The SRBC were removed from the T cells used 0.87% ammonium chloride. All cell preparations were washed twice in medium before use in the LMI test. Cell viabilities were always over 90% by trypan blue exclusion.

The effectiveness of the T-B separation was checked periodically by re-rosetting using neuraminidase treated SRBC for T cells and with chromic chloride treated fresh ox erythrocytes conjugated with anti-human immunoglobulin for cells bearing surface immunoglobulin (which were taken to be equivalent to B cells). This is a modification of the method of Ling et al. (1977). The T-enriched preparation always contained >92% T cells and <5% B cells and the B-enriched preparation >54% B cells (usually >70%) with <10% T cells. Mean recovery of cells from the rosetting procedure was 76%.

LMI test with subpopulations of leucocytes using PPD

This was performed on four of the five controls who were Mantoux tested as described above. Three were Mantoux positive and one negative. All had a standard LMI test with PPD as antigen at a concentration of 200 μ g/ml as well as the test performed with polymorphs, mononuclear cells and polymorphs plus T cells and plus B cells. (In one subject there were insufficient B cells to perform a quadruplicate assay in this last case).

LMI test with subpopulations of leucocytes using
gluten fraction III.

Eight healthy controls and 10 coeliac patients were studied. Five patients were studied untreated and five after treatment with a gluten-free diet (mean duration of GFD 11 months, range 6 - 15 months). As with the parallel experiments using PPD, there were two occasions when insufficient B cells were obtained and three further occasions when, because of poor yield, all the mononuclear cells had to be used in the T-B separation procedure.

Serum incubation

To investigate whether a serum factor might be involved in sensitising leucocytes to gluten, the LMI test was performed with cells obtained by dextran sedimentation from a normal control. These cells were incubated with serum from six of the coeliac patients (2 untreated, 4 treated) at 37⁰C for one hour at a dilution of 1 in 2. The cells were then washed twice in cold medium (to attempt to minimise elution of any cytophilic antibody (Berkin & Benacerraf, 1966) and used in the LMI test with gluten fraction III as usual.

Aliquots of the cells were also pre-incubated with aggregated human IgG in an attempt to block Fc receptors. The IgG (kindly supplied by Mr. P. Guillou,

Department of Surgery, St. James's University Hospital) at 64mg/ml was aggregated by heating to 63⁰C for 20 minutes and then incubated with the cells at 37⁰C for one hour at a final concentration of 5.8mg/ml (this concentration is sufficient to block Fc receptors in an experimental ADCC system (Carnaud et al. 1980)). The cells were then washed twice in cold medium, incubated with the coeliac sera as above, rewashed twice and used in the LMI test.

Jejunal biopsy organ culture studies

General description of technique

Biopsies. All subjects studied were undergoing jejunal biopsy as part of routine diagnostic procedure. Biopsies were taken, with informed consent, from the region of the ligament of Treitz after at least a six-hour fast and after ensuring that there were no significant abnormalities of platelet count or prothrombin time, using the Quinton biopsy apparatus. Multiple biopsies were taken routinely; the most suitable specimens were sent for diagnostic histology and the remainder used for organ culture studies.

Culture medium. The basic culture medium used was that described by Ferguson et al. (1975) and consisted of Trowell's T8 medium (6ml), NCTC 135 medium (2ml), 1M HEPES buffer (0.1ml) (all obtained from Flow

Laboratories Ltd., Irvine), 200mM glutamine (0.2ml) (Sigma Chemicals Ltd.), foetal calf serum (1.5ml) (Wellcome Reagents Ltd.), 100 units penicillin and 100 units streptomycin (Pharmacy, St. James's University Hospital). This medium (the "control" medium) was mixed freshly 1-2 hours before use in the organ culture system and was kept at 37⁰C until the biopsies were obtained.

Culture procedure. The biopsies, delivered by the Quinton apparatus in Ringer-Tyrode solution, were immediately placed in ice-cold control medium and cut into two pieces. The pieces were mounted with the villous surface uppermost on wire grids and placed in sterile organ culture dishes (Falcon Plastics Ltd., suppliers: Becton, Dickinson Ltd., Middlesex). The central well was then filled with the appropriate culture medium so that the medium just touched the under-surface of the biopsy. This required approximately 1ml of medium. Care was taken not to "flood" the biopsy. The time from excision of the biopsy to this point was approximately five minutes. The outer ring of the culture dish contained an absorbent paper ring which was saturated with sterile water or normal saline to produce a humid atmosphere in the culture dish.

The dishes were then placed in a sterile anaerobic jar which was gassed with 95% oxygen and 5% carbon dioxide for 30 minutes, sealed and incubated at 37°C.

Jejunal biopsy and lymphocyte co-culture

Thirteen patients with treated coeliac disease and five controls were studied. The patients (7 men, 6 women, mean age 35 years, range 22 - 55 years) had all been on gluten-free diets for at least six months (mean 4.9 years, range 6 months - 10 years). The controls were from the "biopsied control" group. All had histologically normal jejunal biopsies. Final diagnoses were irritable bowel syndrome (2), Crohn's disease (1), food allergy (1) and dietary iron deficient anaemia (1).

Lymphocyte preparation

Peripheral blood mononuclear cells were prepared from 20ml of heparinised venous blood taken on the morning of the biopsy by centrifugation through Histopaque 1077 as described above. Cells were washed twice in Medium 199 and finally suspended at a concentration of 5×10^6 cells per ml. 0.2ml of this suspension was added to organ cultures to give 1×10^6 lymphocytes per culture. Organ cultures without added lymphocytes had 0.2ml of Medium 199 added. Lymphocyte viability was tested by trypan blue exclusion.

Organ culture

Organ cultures of mucosal biopsies were set up as described above. Duplicate cultures were set up in control medium, in control medium with added lymphocytes, in medium containing gluten fraction III at 1mg/ml and in medium containing gluten fraction III with added lymphocytes. Cultures were terminated at 24 hours. One specimen was taken for quantitative histology and the other for biochemical analysis. A biopsy which had not been cultured was included in each case and was processed with the post-culture specimens.

Quantitative histology

Objective assessment of histological change was made by measurement of mean enterocyte height, using the method described by Howdle et al. (1981). All slides were coded and randomised and all measurements were made by a single observer. The sections were projected onto a screen using a Leitz microscope projector at a magnification such that 1mm on the projected image corresponded to 1μ on the section. The heights of at least 20 epithelial cells chosen randomly from throughout the section were then measured from the epithelial side of the basement membrane to the outer edge of the brush border using a perspex ruler. Cells measured were those with central, basally-placed

nuclei. Areas which were tangentially sectioned or poorly preserved were avoided as were cells near crypts or the tips of villi. The mean cell height was then calculated. The precision of this measurement was assessed by calculating the coefficient of variation for the 20 measurements made on each section. Results from 10 sections chosen at random and measured by one observer are shown in Table 6. The mean coefficient of variation for these individual results is 6.77% which is very close to the 6.6% calculated from data of Marsh (1972). The reproducibility of the assay was assessed by measurement of 10 coded sections (from both coeliacs and controls) in random order on two separate occasions by a single observer. These results are given in Table 7. The calculated standard deviation for duplicates from these results is 0.458μ . It can thus be calculated that in this assay a difference in results of $0.458 \times \sqrt{2} \times 1.96 = 1.27\mu$ is unlikely to be due to observer error with a probability level of $p < 0.05$: (Campbell & Owen, 1967).

Biochemical analysis

Specimens for biochemical analysis were homogenised in 2ml of 0.9% saline with ten strokes of a teflon pestle in a glass homogeniser (Thomas Co.U.S.A.) and were then stored at -70°C before being assayed for

TABLE 6

Variability of mean enterocyte height measurement
on individual sections

<u>Mean cell height(μ)</u>	<u>S.D.</u>	<u>C.V.(%)</u>
30.85	1.76	5.71
28.85	1.66	5.75
28.30	1.66	5.87
25.20	2.09	8.29
26.4	2.44	9.24
31.77	1.96	6.17
24.10	1.62	6.72
26.35	1.17	4.44
23.14	1.96	8.47
22.80	1.61	7.06

Mean CV = 6.77%

TABLE 7

Enterocyte height measurements made by the same observer on 10 sections on two separate occasions

<u>Section</u>	<u>1st measurement</u> <u>(μm)</u>	<u>2nd measurement</u> <u>(μm)</u>	<u>Difference</u>
1	19.84	19.63	+ 0.21
2	26.79	27.52	- 0.73
3	21.22	21.25	- 0.03
4	26.70	26.10	+ 0.60
5	28.04	28.65	- 0.61
6	19.95	20.53	- 0.58
7	24.67	24.95	- 0.28
8	28.00	28.29	- 0.29
9	24.48	25.85	- 1.37
10	19.57	20.24	- 0.71

$$SD = \sqrt{\frac{\sum d^2}{n}} = 0.458$$

alkaline phosphatase activity, protein content and DNA. Enzyme activities were then expressed either per mg wet weight of biopsy, per mg of protein or per μg of DNA.

Alkaline phosphatase assay

Alkaline phosphatase was estimated by the fluorimetric method described by Peters et al. (1972) using 4-methyl umbelliferol phosphate (Sigma Chemicals Ltd.) as substrate at pH 8.7 with 0.05M borate buffer containing 5mM MgCl_2 . Assays were performed in duplicate at a dilution of 1 in 20 (determined as suitable by preliminary experiments) with solutions of methyl umbelliferone (Sigma) of 1, 5 and 10nmol/ml as standards (emission of standard solutions was shown to be linear up to 50nmol/ml in preliminary experiments). Both enzyme and substrate blanks were included in each assay. Incubation period was 15 minutes and the reaction was stopped by addition of sodium/glycine buffer at pH 10.5. Fluorescence was read on an Aminco-Bowman Spectrophotofluorimeter (American Instrument Co.) at Excitation 365nm and Emission 460nm.

As it was impracticable to assay all specimens for alkaline phosphatase fresh the effect of both freezing and length of storage was investigated. Data were available on 76 pairs of homogenates which had been

assayed by the above technique both fresh and after a single freezing with storage for a varied period. Results show that for specimens measured in less than seven days there is no significant deterioration; those measured in seven days or less just fail to show significant deterioration whereas those measured after seven days do deteriorate significantly. Analysis of 29 pairs of results on samples analysed after one or two freeze-thaw cycles show a significant deterioration after the second freeze-thaw both for the whole group and for 12 samples stored for less than seven days (Howdle, personal communication). All results in this thesis are from samples stored for less than seven days and analysed after a single freeze-thaw cycle. Results are expressed as milliunits, 1mU corresponding to the hydrolysis of 1nmol of substrate per minute per mg of protein in the same homogenate. Within batch variation was estimated on five biopsies which were assayed with at least eight replicates. Coefficients of variation of these assays ranged from 5.0 to 11.2% which was regarded as acceptable.

Protein assay

Protein was assayed in the biopsy homogenate using the method of Lowry et al. (1951) using solutions of bovine serum albumin (BDH Ltd.) as standards. The

effect of storage was estimated on 29 samples which had been assayed twice with storage periods ranging from four days to 15 months. Results are shown in Figure 8, which shows the difference between the two assays plotted against time. There is no trend for the differences to vary from zero with increasing time of storage and it was concluded that the assay was unaffected by storage at -70°C . Within batch variation was estimated on 12 replicates of a single homogenate and gave a coefficient of variation of 2.9%.

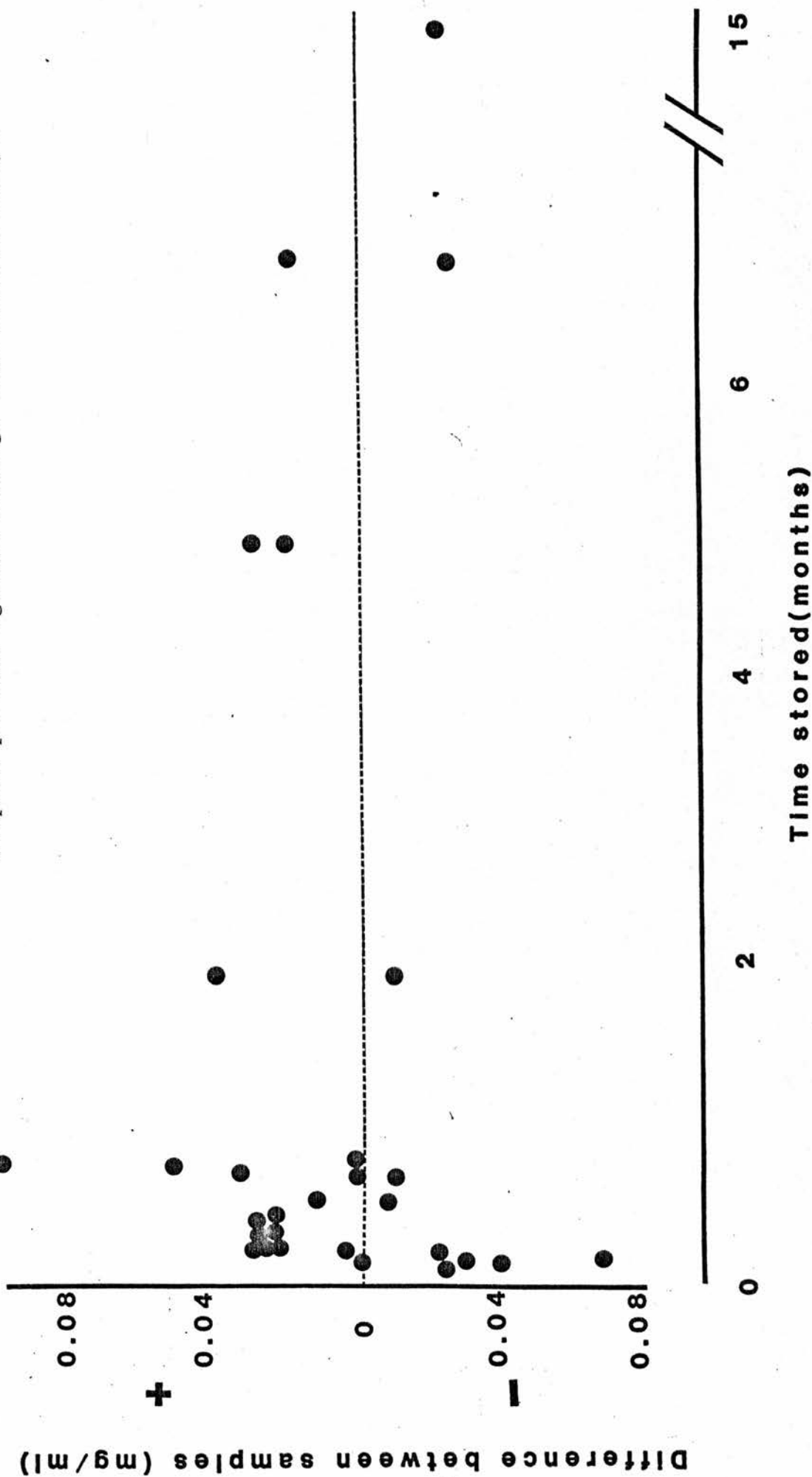
DNA assay

DNA was measured using the fluorescent method of Le Pecq & Paoletti (1966).

Phosphatidyl inositol turnover assay (I)

A method of assaying the incorporation of tritiated myoinositol into cellular lipids as a measure of phosphatidyl inositol turnover in cultured jejunal biopsies was developed. This was based on the method described by Ogmundsdottir & Weir (1979). A total of 31 patients undergoing routine jejunal biopsy for diagnostic purposes was studied. Fifteen had normal biopsies ("biopsied controls"), 10 were untreated coeliacs and six treated coeliacs. Biopsies were taken and set up in organ culture as described above, with and without the addition of gluten fraction III to the

FIG. 8. Differences between repeated protein assays on stored samples plotted against storage time between assays



culture medium at a concentration of 1mg/ml, and with the addition of 2 μ Ci of 3 H-myoinositol (Radiochemical Centre, Amersham) in PBS. The basis of the assay used is simply extraction of all the lipid material in the tissue studied. This lipid is then dissolved in an organic scintillation fluid for counting. As the labelled myoinositol is incorporated specifically into phosphatidyl inositol no subfractionation of the lipid is required. The method described by Ogmundsdottir & Weir (1979) was designed for use with macrophage monolayers and thus had to be modified for use with organ culture of jejunal biopsies. Preliminary experiments showed that the short culture periods (after adding the label) used with cell monolayers gave poor incorporation of label using biopsies, perhaps largely as a result of the relatively poor access of label in the medium to the enterocytes of the surface of the biopsy, which itself is floating on the surface of the medium. To minimise disturbance of the organ culture system, the labelled myoinositol was thus incorporated into the medium from the start of the culture period, which was 24 hours. At the end of this time the cultures were terminated by immersing the biopsies into ice-cold phosphate buffered saline (PBS, pH 7.2). The biopsies were then washed thoroughly in

cold PBS (5 changes of 5ml aliquots of PBS were used as preliminary studies had demonstrated minimal radioactivity extracted into the PBS after this number of changes). On the first 13 patients studied (7 biopsied controls, 4 untreated coeliacs and 2 treated coeliacs) the following procedure was then adopted. Each washed biopsy was mopped dry, weighed and then transferred to a glass homogeniser tube and 5ml of a 2:1 chloroform-methanol mixture added. The biopsy was then homogenised using ten strokes of a teflon pestle as described above. The lipids were then extracted from this homogenate by incubation at 37°C for 20 minutes with vigorous shaking on a Whirlimixer every five minutes. Two ml of liver lipid extract (prepared as described below) and 3ml of 0.5M MgCl₂ were then added and mixed well. Addition of a lipid carrier reduces loss of labelled lipid during extraction (Ogmundsdottir & Weir, 1979) whilst MgCl₂ reduces the loss of polar lipids into the aqueous phase (Folch et al. 1957). After mixing, the phases were then separated by centrifugation at 1000 rpm for 10 minutes. The aqueous phase was then aspirated with a Pasteur pipette and discarded and the organic phase equilibrated for 10 minutes against 3ml of 0.5M MgCl₂ without mixing. The aqueous phase was once more discarded and the organic phase carefully transferred into disposable liquid

scintillation vials, taking care not to carry over any droplets of the aqueous phase. The organic phase was then evaporated to dryness at 45⁰C under a stream of nitrogen. The dried extract was then dissolved in a toluene based scintillation fluid containing 0.4% 2,5 diphenyloxazole (POP) and 0.01% 1,4 di (2,5 phenyloxazole) benzene (POPOP) (both from BDH Ltd.). Radioactivity was then counted for 10 minutes in a Packard Tri-Carb Liquid Scintillation Spectrophotofluorimeter Model 3375.

Extraction of lipid carrier

Twenty five grams of rat liver were homogenised in 50ml of a 2:1 chloroform-methanol mixture and extracted at 37⁰C for 20 minutes. Twenty five ml of 0.5M MgCl₂ was added and thoroughly mixed. The mixture was then centrifuged at 300xg for 10 minutes. The aqueous phase was discarded and the organic phase withdrawn from beneath the compacted liver tissue with a Pasteur pipette. The organic phase was then equilibrated for 10 minutes with 0.5M MgCl₂ without mixing, spun at 300xg for 10 minutes and the aqueous phase discarded. The organic phase was dried with a little anhydrous Na₂SO₄ and evaporated to dryness at 45⁰C under a stream of N². The residue was then dissolved in 50ml of 2:1 chloroform-methanol and stored in 1ml aliquots at

-20⁰C. For use in the lipid extraction this solution was diluted one part in 10 with 2:1 chloroform-methanol.

Reproducibility of assay

The above assay was used on biopsies from 13 patients. In each case the organ cultures with and without added GFIII were performed in duplicate. Results were expressed as counts per minute per mg of tissue (cpm/mg) in the first instance. Results are shown in Appendix Table 1. The results are obviously extremely variable and the coefficient of variation from duplicates is 87.1%. Even when the seven most obviously abnormal results are excluded, the C.V. is 26.5% which is clearly unacceptable.

There were two main reasons for this variability.

Firstly, perhaps because of the low surface tension of the chloroform-methanol, it was extremely difficult to adequately homogenise biopsies in this medium and splashing and loss of solute were common and almost impossible to avoid. Secondly, the wet weight of the biopsy was difficult to measure with any precision as in order to remove all visible surface fluid from the biopsies the mopping technique (using blotting paper) tended to remove a variable amount of surface mucus. The method was thus altered to remove these sources of error.

PI turnover assay (II)

The biopsy cultures were set up and terminated as described above. The biopsies were then homogenised in 2ml of normal saline. Aliquots of 0.5ml of homogenate were then incubated at 37⁰C for 20 minutes after addition of 2.5ml of chloroform-methanol and the lipid extraction then proceeded as described above. The remaining homogenate was stored at -70⁰C for later protein estimation, which was performed by the Lowry technique. Radioactive counts were converted into disintegrations per minute (dpm) by use of a standard Quench curve (see below) and results expressed as dpm/mg protein.

Reproducibility of assay

This assay was used on samples from 18 patients. Eight were biopsied controls, six untreated coeliacs and four treated coeliacs. One sample was spilt and a duplicate assay could thus not be performed. There are thus 70 pairs of duplicate observations available and these are shown in Appendix Table 2. The calculated C.V. for duplicates in these results is 7.7% which was felt to be acceptable.

Quench curve

This was derived using a ³H standard (LKB-Wallace Internal Standard Kit, LKB Instruments Ltd., Croydon)

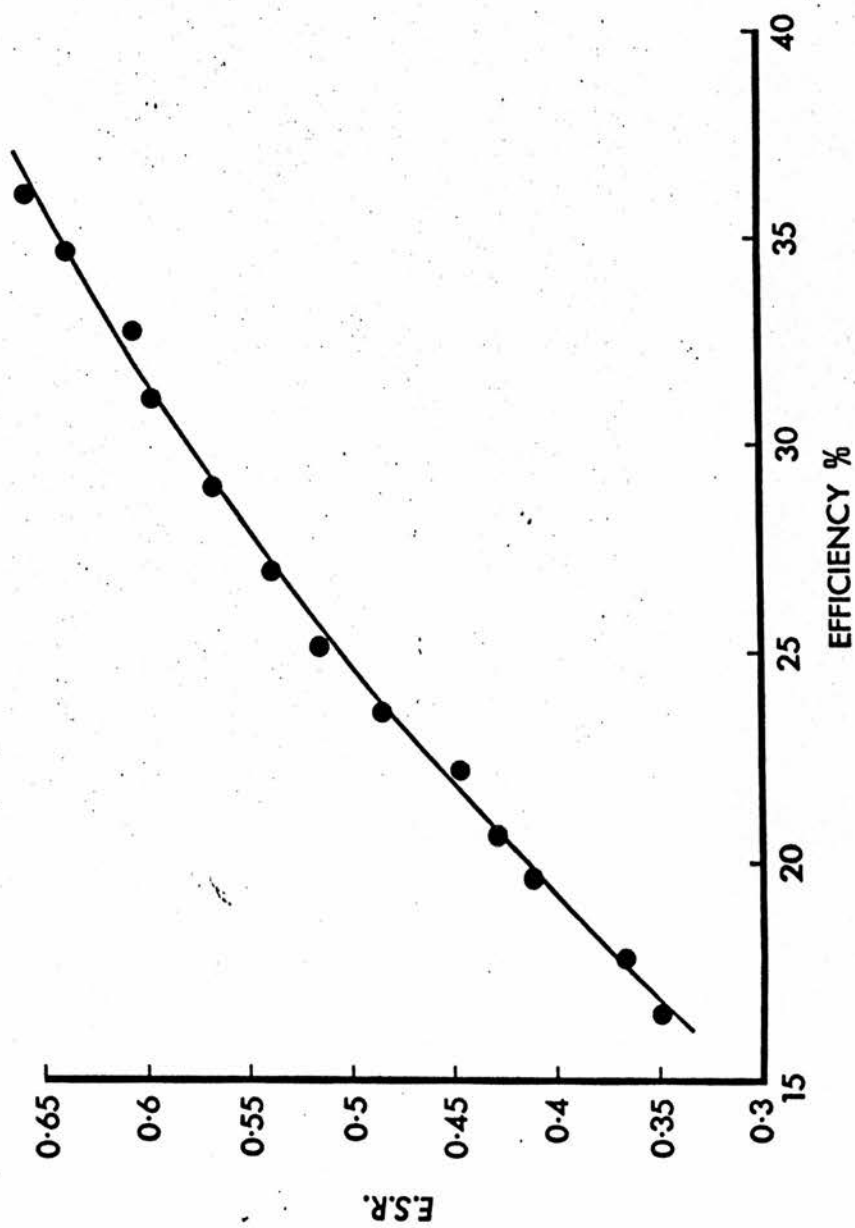


FIG. 9. Quench curve for PI turnover assay

by sequential additions of 1 μ l aliquots of carbon tetrachloride using the scintillation fluid described above. The tritium standard was rated as producing 209,500 dpm. This figure was adjusted for decay using the formula:

$$N = N_0 \exp \left(\frac{0.693t}{t} \right)$$

Results are shown in Appendix Table 3 and Figure 9. Use of this curve enabled results expressed as counts per minute to be converted to disintegrations per minute allowing for the efficiency of counting.

R E S U L T S

"One should always be a little improbable"

Phrases & Philosophies for the Use of the Young
Oscar Wilde, 1894

LMI test with gluten fraction III - influence of
HLA-B8 and diet

Results of the LMI test in 30 normal controls with gluten fraction III as antigen are shown in Figure 10. Fourteen of the controls had the histocompatibility antigen HLA-B8 and 16 did not. Those having HLA-B8 had a mean migration index of 0.88 (range 0.78 - 1.02; SD 0.08). Those lacking HLA-B8 had a mean migration index of 0.98 (range 0.80 - 1.17; SD 0.10). Using Student's t test, there is a significant difference between these groups ($p < 0.02$) indicating significantly greater immunity in the HLA-B8 group, though there is a great overlap.

Figure 11 shows the results of the LMI test with gluten fraction III in 27 untreated coeliacs divided into 22 having HLA-B8 and five lacking this antigen, compared with the 14 HLA-B8 controls. Mean migration index for the HLA-B8 coeliacs was 0.88 (range 0.66 - 1.14; SD 0.12) and for the non-HLA-B8 coeliacs 0.91 (range 0.75 - 1.14; SD 0.15). Neither of these groups differ significantly from the HLA-B8 controls (though they do when compared with the non-HLA-B8 controls, nor do they differ from each other.

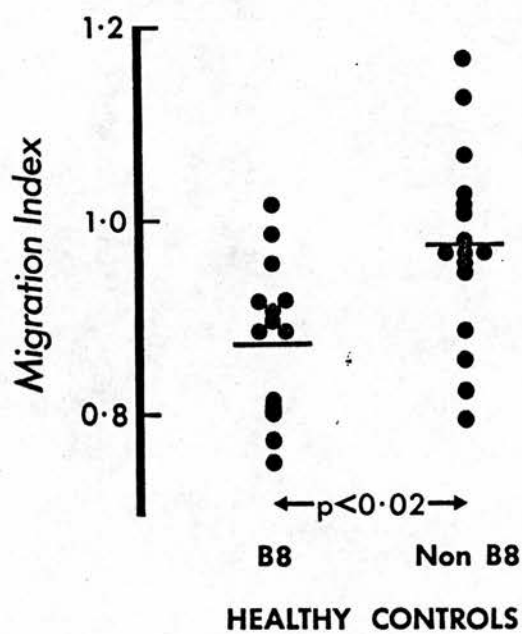


FIG. 10. Migration indices to gluten fraction III in 30 healthy controls divided according to the presence or absence of HLA-B8

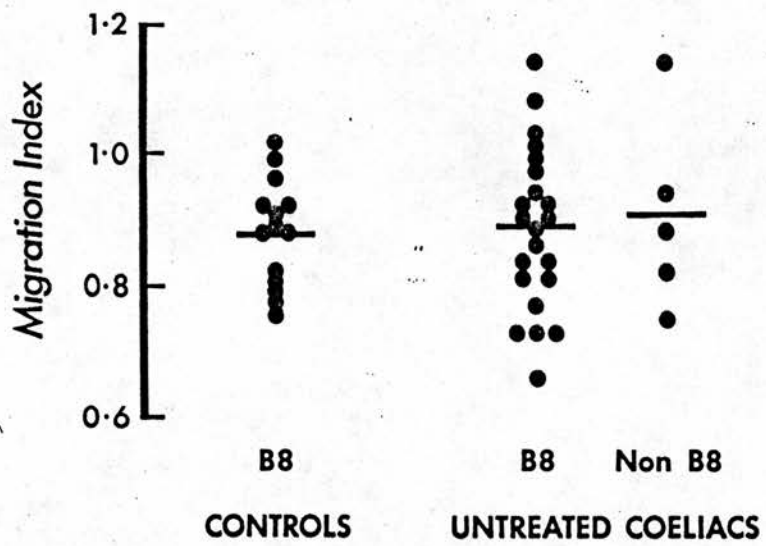


FIG. 11. Migration indices to gluten fraction III in 27 untreated coeliacs divided according to possession of HLA-B8 compared with 14 normal controls having HLA-B8

Figure 12 shows the results of the LMI test for all the coeliac patients studied divided according to HLA-B8 status and by treatment status. Coeliacs early in treatment (gluten-free diet for 3-12 months) had significantly lower migration indices than the untreated group (B8: mean 0.79, SD 0.06; non-B8: mean 0.76, SD 0.05, $p < 0.01$). The group on treatment for over 12 months ("late treated") showed a significant increase in migration indices (B8: mean 0.88, SD 0.09; non-B8: mean 0.88, SD 0.06, $p < 0.01$) and gave values indistinguishable from HLA-B8 controls or from untreated coeliacs. It is noteworthy that coeliacs having HLA-B8 and those lacking HLA-B8 give similar results at all stages and respond similarly to changes in dietary status.

Results from the six coeliac patients studied at all stages of treatment are shown in Figure 13 and show a similar pattern of a fall in migration index early in treatment and a later rise. This holds for B8 and non-B8 individuals.

Influence of DR3

Results from the 20 normal controls typed for the DR3 histocompatibility antigen are shown in Figure 14. The seven subjects with DR3 gave a mean migration index of 0.89 (range 0.78 - 1.02, SD 0.08); the 13 lacking

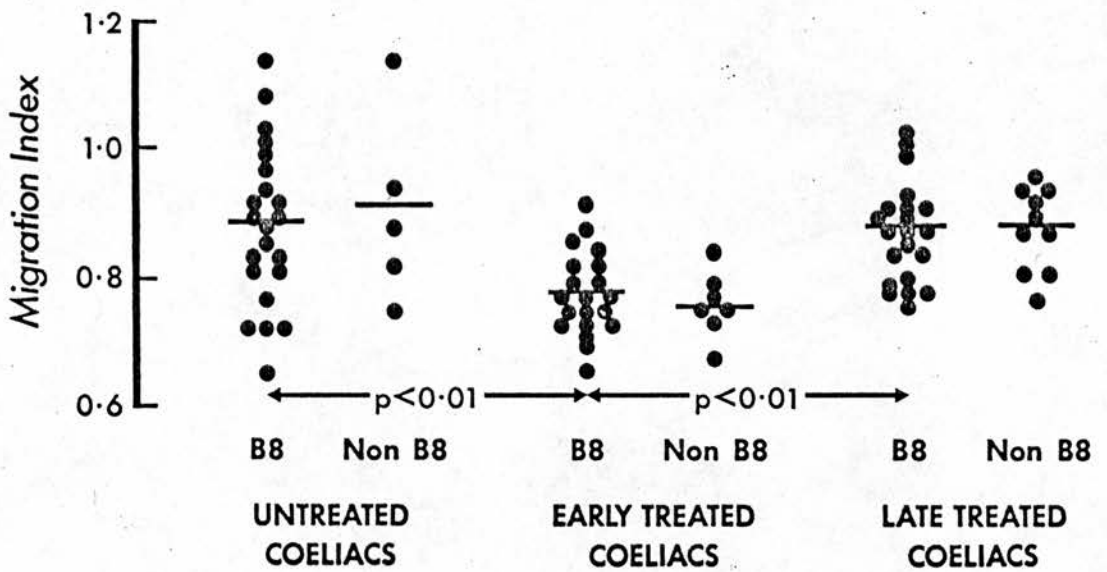


FIG. 12. Migration indices to gluten fraction III in 58 coeliacs divided according to HLA-B8 status and dietary status.

('Early treated' = gluten free diet for 3 to 12 months

'Late-treated' = gluten free diet for over 12 months)

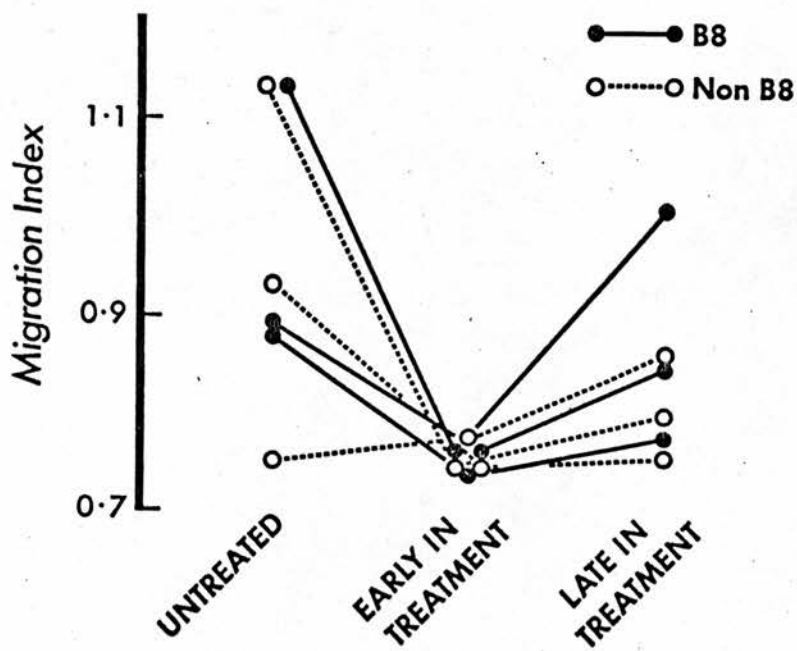


FIG. 13. Migration indices to gluten fraction III in 6 patients studied sequentially at all stages of treatment

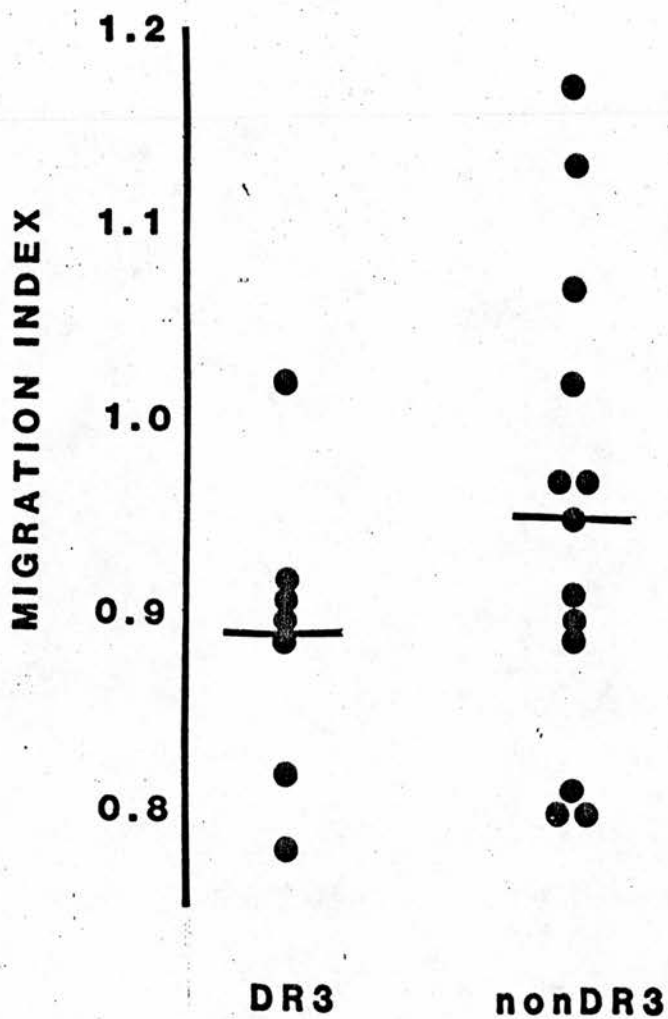


FIG. 14. Migration indices to gluten fraction III in 20 normal controls divided according to possession of the DR3 antigen

DR3 gave a mean migration index of 0.95 (range 0.80 - 1.17, SD 0.12). The DR3 group thus have a lower mean value but do not significantly differ statistically from the non DR3 group.

LMI test with other dietary antigens

Results of the LMI test in controls and coeliacs (sub-divided by dietary status and HLA-B8 status) to the dietary antigens egg, bovine serum albumin and milk are shown in Figures 15 - 17. Considering the results from the normal controls first, it can be seen that the possession or lack of HLA-B8 does not seem to influence migration indices to egg (mean HLA-B8 0.93, non-B8 0.98), to BSA (mean HLA-B8 0.97, non-B8 1.02) or milk (mean HLA-B8 0.97, non-B8 0.98). In all three cases there is no statistically significant difference between the HLA-B8 and non-HLA-B8 groups. Furthermore, though the numbers of HLA-B8 negative coeliacs were too low to allow valid statistical comparison with HLA-B8 coeliacs, the responses of the two groups were similar for each antigen at each stage of treatment. HLA-B8 status was thus disregarded in subsequent statistical analysis of results for egg, BSA and milk antigens.

With egg antigen there is a significant difference between migration indices of controls (mean 0.96) and untreated coeliacs (mean 0.84, $p < 0.01$ by Wilcoxon's

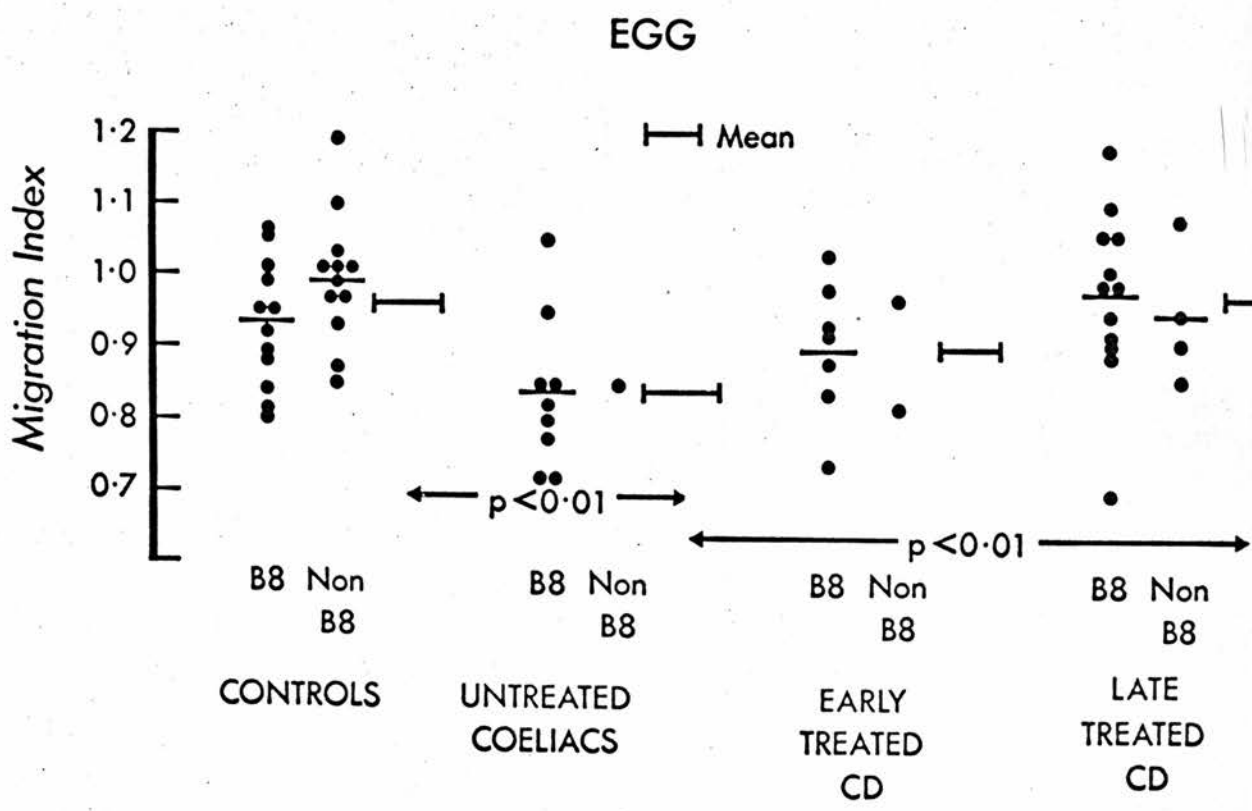


FIG. 15. Migration indices to egg antigen in controls and coeliacs at various stages of treatment divided according to possession of HLA-B8

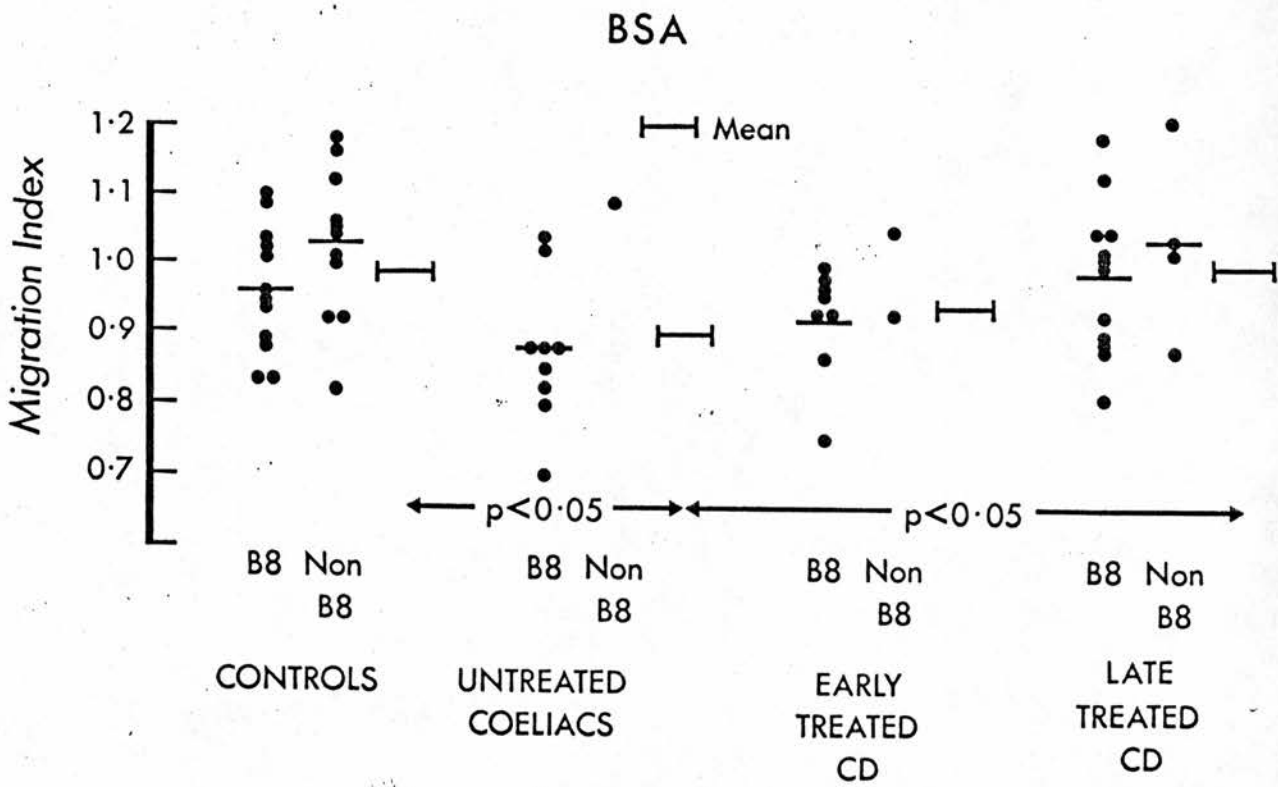


FIG. 16. Migration indices to bovine serum albumin in controls and coeliacs at various stages of treatment divided according to possession of HLA-B8

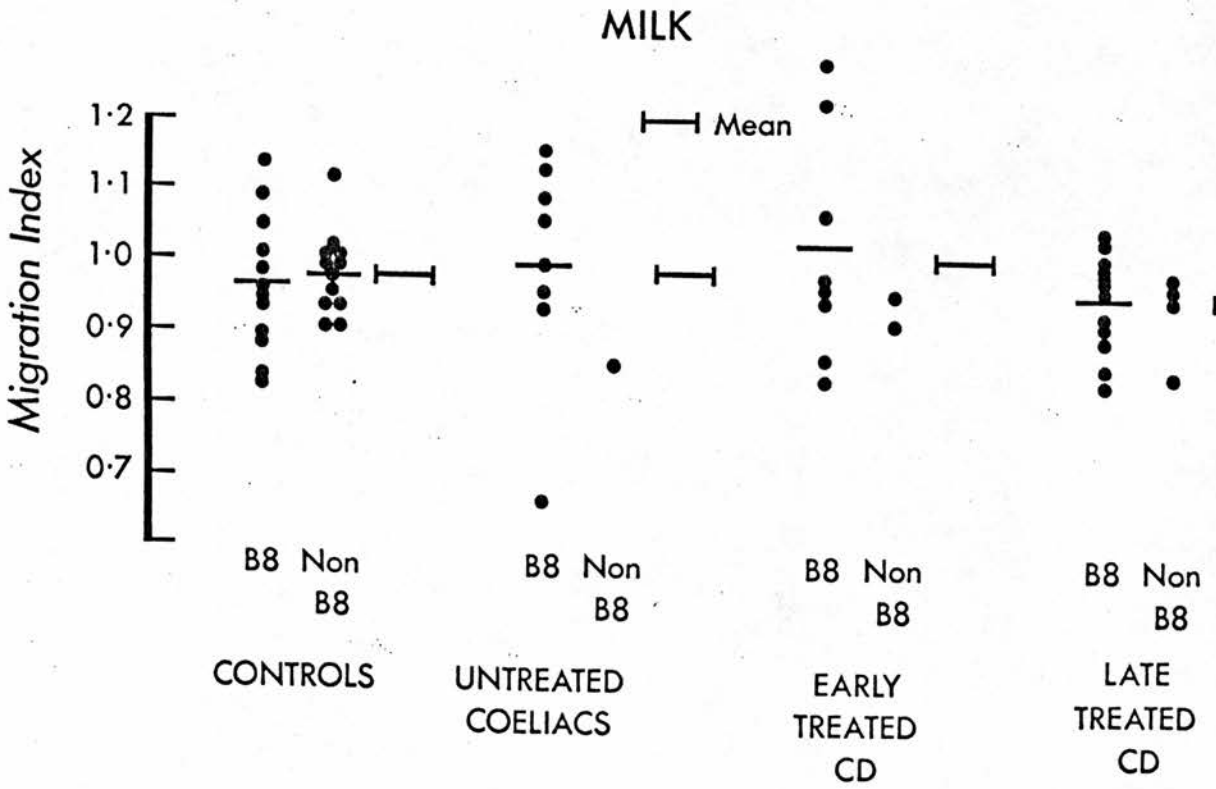


FIG. 17. Migration indices to milk antigen in controls and coeliacs at various stages of treatment divided according to possession of HLA-B8

rank sum test). The late treated group was indistinguishable from controls (mean 0.97) and also differed significantly from the untreated group ($p < 0.01$). The early treated group occupied an intermediate position (mean 0.90).

Results with BSA were similar: untreated coeliacs (mean migration index 0.90) differed from controls (mean 0.99, $p < 0.05$ by Wilcoxon's rank sum test) and from patients treated for over one year (mean 1.00; $p < 0.05$) with the early treated group in an intermediate position (mean 0.94).

In the case of the milk antigen, there were no significant differences between the groups, mean migration indices being: controls 0.98; untreated coeliacs 0.99; early treated coeliacs 1.01; late treated coeliacs 0.96.

The same subjects were also studied with gluten fraction III and results are shown in Figure 18. Here a different pattern is seen with significantly lower migration indices in the HLA-B8 normal controls (mean 0.88) compared with controls lacking this antigen (mean 0.98, $p < 0.05$ by Wilcoxon's test). Untreated coeliacs (mean 0.87) differed significantly from HLA-B8 negative controls ($p = 0.05$) but not from the HLA-B8 controls. Coeliacs early in treatment had a mean migration index

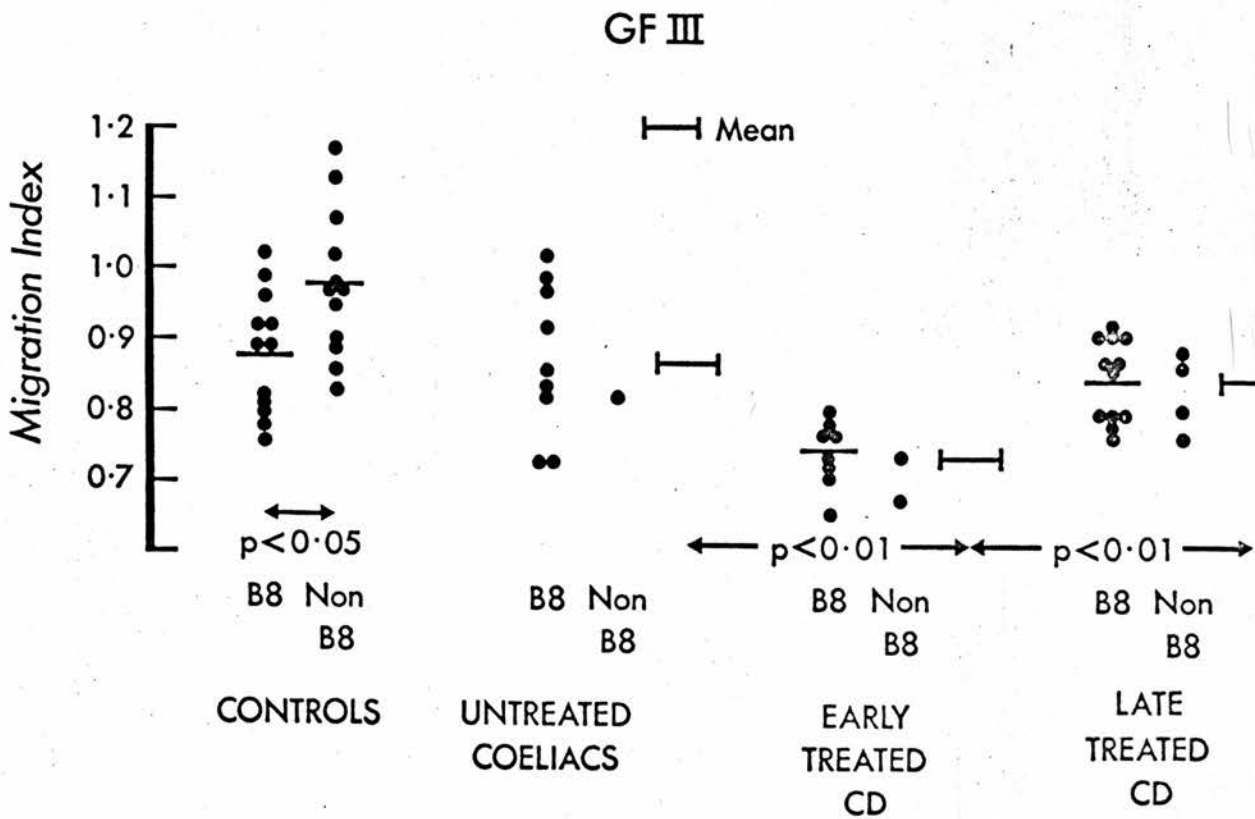


FIG. 18. Migration indices to gluten fraction III in those controls and coeliacs included in studies with other dietary antigens

of 0.75, which differed significantly from both untreated coeliacs ($p < 0.01$) and the late treated group (mean 0.85, $p < 0.01$). HLA-B8 and non-HLA-B8 coeliacs gave similar results in each group. These results are in close accord with those presented above (see Figures 10 - 12).

Figure 19 illustrates the migration indices to each of the four dietary antigens studied for the 10 untreated coeliacs. The line at migration index 0.80 represents the level below which it is conventionally accepted that definite cell-mediated immunity is demonstrated. This convention in fact agrees well with the normal ranges (calculated as mean \pm 2SD) derived from the results of the normal control group for all antigens, except in the case of gluten fraction III where the influence of HLA-B8 affects the "normal" range. Using this conventional criterion, four of the 10 coeliacs have cellular immunity to egg antigen, two to BSA, one to milk and two to gluten fraction III.

Jejunal biopsies. Results of grading of jejunal biopsies by stereomicroscopic appearance are shown in Table 8 and by histological appearance in Table 9. There is a clear improvement in morphology by both criteria in the early treated group compared with the untreated patients. The late treated group show further improvement.

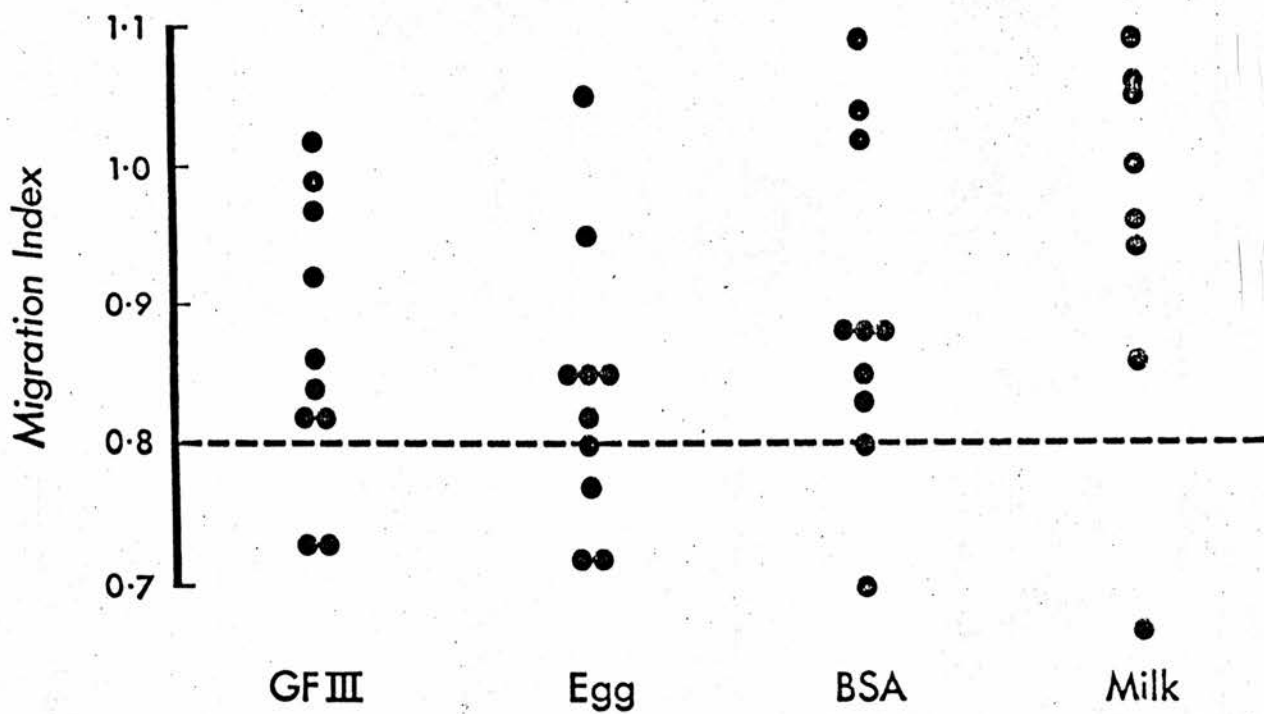


FIG. 19. Migration indices to four dietary antigens in 10 untreated coeliac patients

TABLE 8

Stereomicroscopic grading of jejunal biopsies

	Untreated coeliacs (n = 10)	Early treated coeliacs (n = 8)	Late treated coeliacs (n = 11)
Flat	9	0	0
Convolutated	1	2	0
Ridged	0	4	1
Villous	0	2	10

TABLE 9

Histological grading of jejunal biopsies
(SVA = subtotal villous atrophy.
PVA = partial villous atrophy)

	Untreated coeliacs (n = 10)	Early treated coeliacs (n = 8)	Late treated coeliacs (n = 11)
SVA	5	0	0
Severe PVA	5	4	1
Mild PVA	0	3	4
Normal villi	0	1	6

Comparison of normal control and biopsied control groups

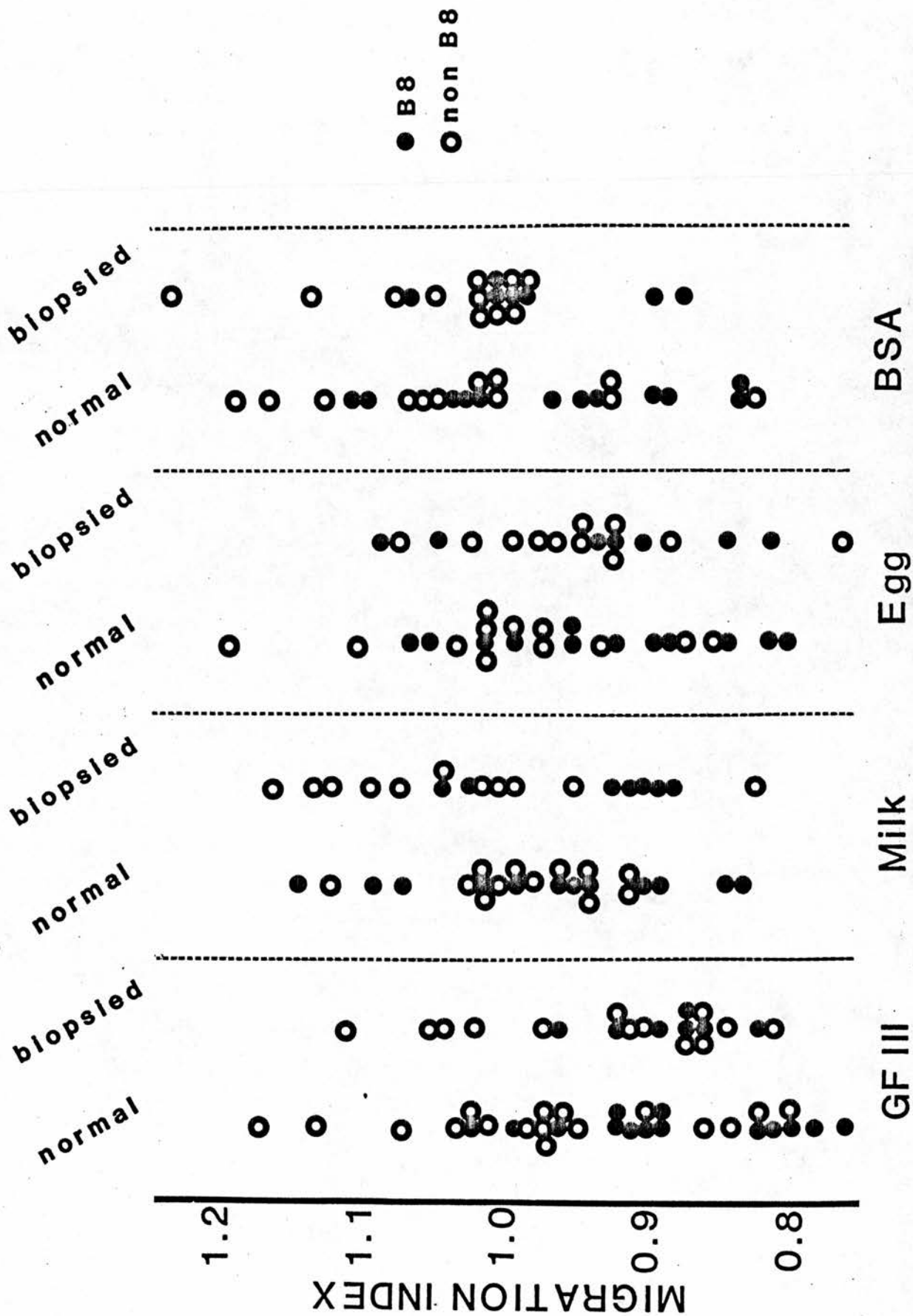
Figure 20 illustrates the comparison of results to all four dietary antigens between the group of normal controls and the group of biopsied controls. The two groups give similar results for all antigens. The only notable difference is the lack of a statistically significant difference between HLA-B8 biopsied controls (mean 0.88) and non-HLA-B8 biopsied controls (mean 0.94) with gluten fraction III as antigen, though the trend towards lower values in the HLA-B8 group is still evident.

Comparison of two subfractions of gluten as antigens in the LMI test

Figure 21 shows the results of the LMI test using the B2 subfraction of gluten in the six controls and nine coeliacs studied. Mean migration index for the control group was 1.02 (range 0.87 - 1.09), for the five untreated coeliacs 0.99 (range 0.92 - 1.04), for the four treated coeliacs 1.04 (range 0.90 - 1.17) and for the coeliac group as a whole 1.01. There is thus no discrimination between these groups with this antigen in this system.

All 15 subjects were studied with gluten fraction III and the correlation of results with B2 and with gluten fraction III is shown in Figure 22. With

FIG. 20. Migration indices to 4 dietary antigens in normal controls and biopsied controls



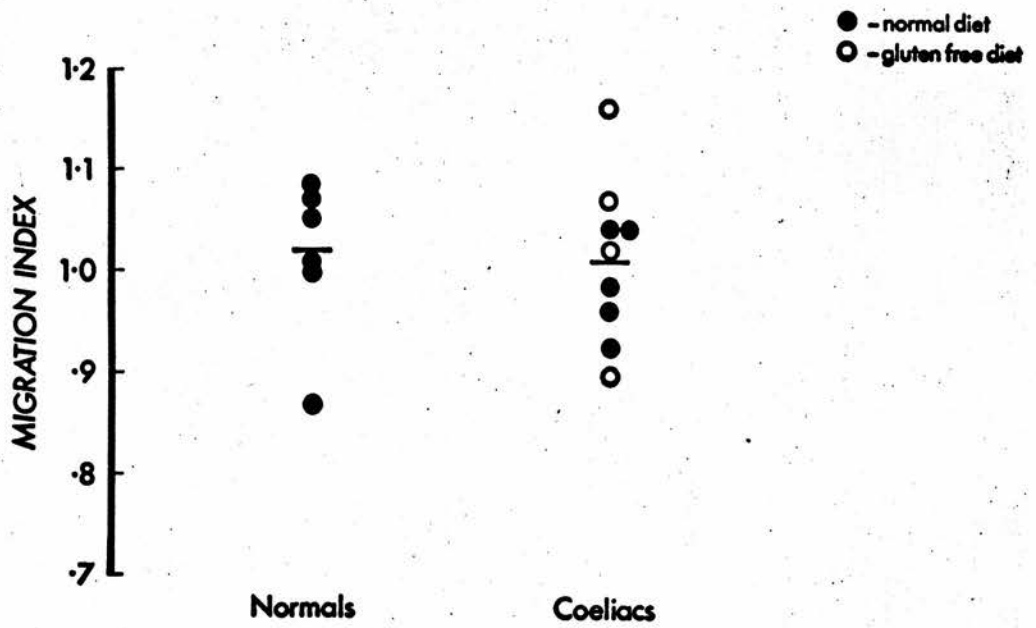


FIG. 21. Migration indices to the B2 gluten subfraction in normal controls and coeliac patients

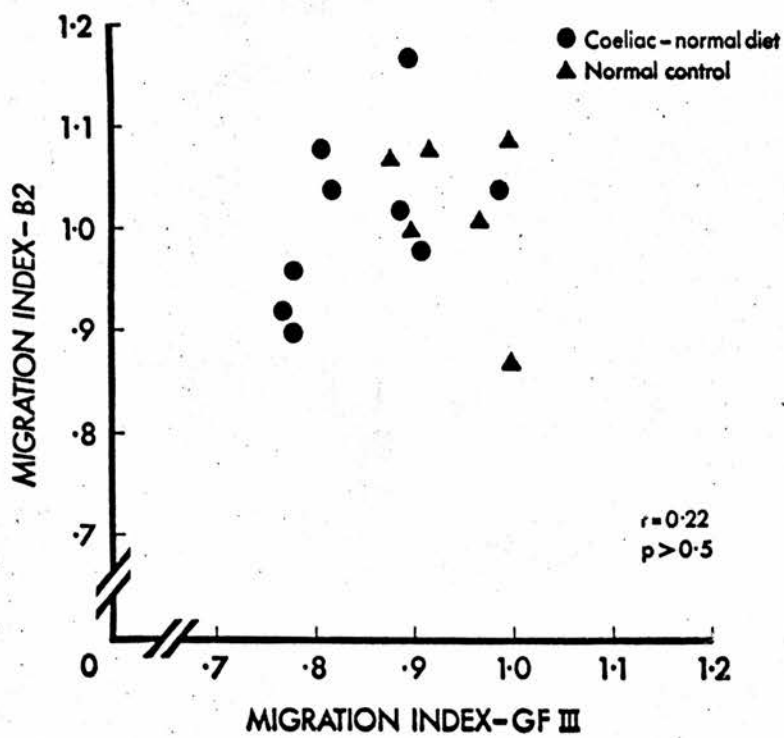


FIG. 22. Correlation between migration indices to gluten fraction III and B2 subfraction in normal controls and coeliacs

gluten fraction III results were compatible with those reported above, mean migration indices being: controls 0.95, untreated coeliacs 0.85, treated coeliacs 0.85. There is no correlation between B2 and GFIII results ($r = 0.22$, $p > 0.5$).

Effect of rhamnose on LMI test

The effect on migration index to gluten fraction III of the incorporation of 5mmol rhamnose is shown in Figure 23. Mean migration index with gluten fraction III alone was 0.79 and with rhamnose and gluten fraction III 0.81. There was no significant change in migration index for any individual nor for the group as a whole.

Migration studies with rhamnose alone showed no effect of rhamnose alone on leucocyte migration, mean migration index being 1.03 (range 0.97 - 1.10).

Effect of puromycin on LMI test

1) Effect of puromycin alone

The effect of puromycin alone on leucocyte migration is shown in Figure 24. Migration areas for both normal controls and coeliac patients are similar whether or not puromycin at 15 μ g/ml is present in the culture medium.

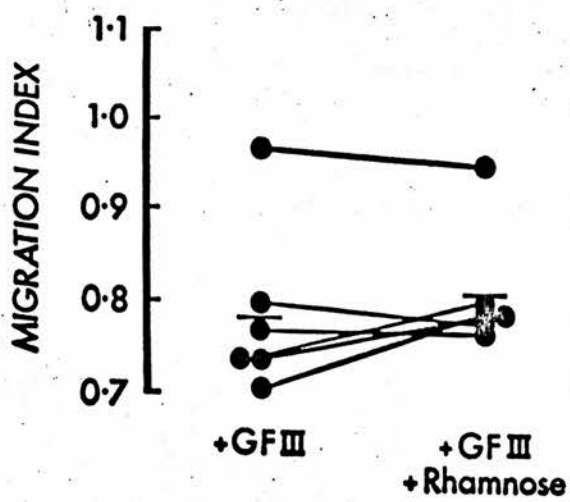


FIG. 23. Effect of 5mmol rhamnose on migration index to gluten fraction III in 6 coeliac patients

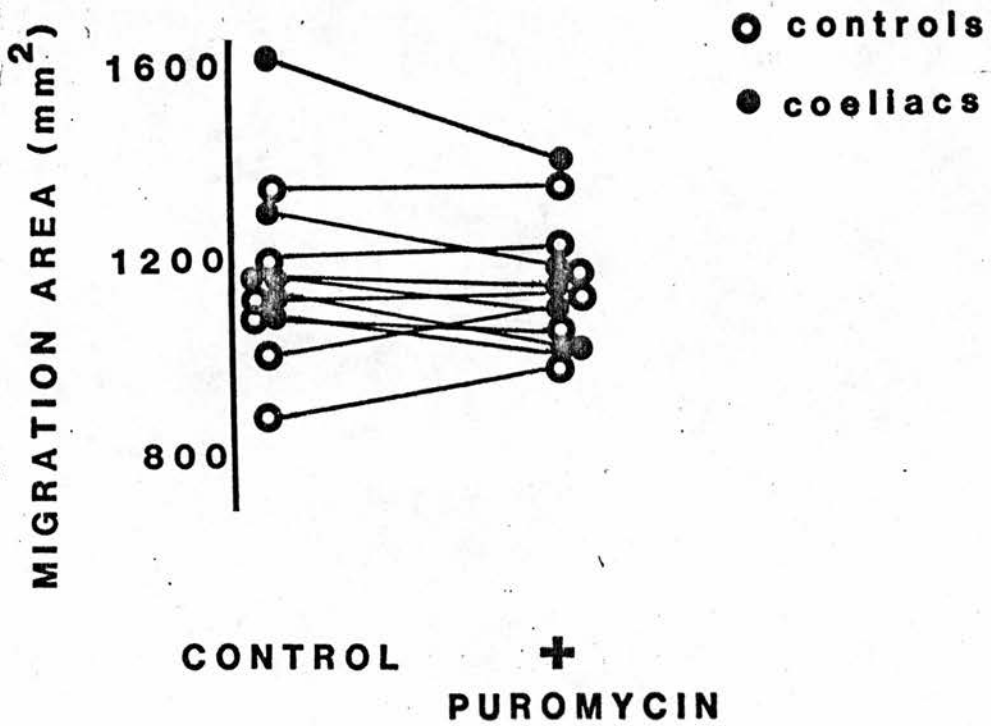


FIG. 24. Effect of puromycin at 15 μ g/ml on leucocyte migration areas in normal controls and coeliacs

2) Effect on the LMI test with PPD

Of the five normal subjects tested, three proved to be Mantoux-positive and two Mantoux-negative. Results of the LMI test with PPD as antigen, with and without incorporation of puromycin into the culture medium, are shown in Figure 25. In the absence of puromycin all three Mantoux-positive subjects show inhibition of leucocyte migration by PPD (migration indices less than 0.8) whilst the Mantoux-negative subjects have migration indices approximating to unity, i.e. there is no migration inhibition. The results in the Mantoux-positive subjects show a marked increase in migration index to PPD in the presence of puromycin, that is, inhibition of leucocyte migration by PPD seems to be blocked by puromycin.

3) Effect on the LMI test with GFIII

The effect of puromycin on migration indices to gluten fraction III in six normal controls and six coeliacs (one untreated, five on a gluten-free diet) is shown in Figure 26. In contrast to the results with PPD there is no trend towards an increase in migration index to approach unity when puromycin is included in the culture medium.

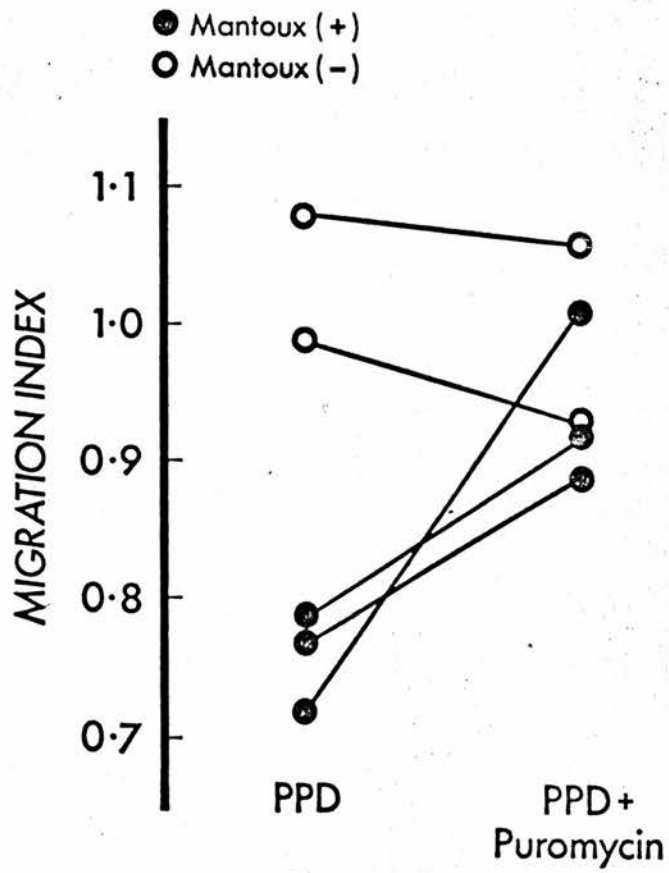


FIG. 25. Migration indices to PPD in 3 Mantoux positive and 2 Mantoux negative subjects with and without the addition of puromycin at 15 μ g/ml to the culture medium

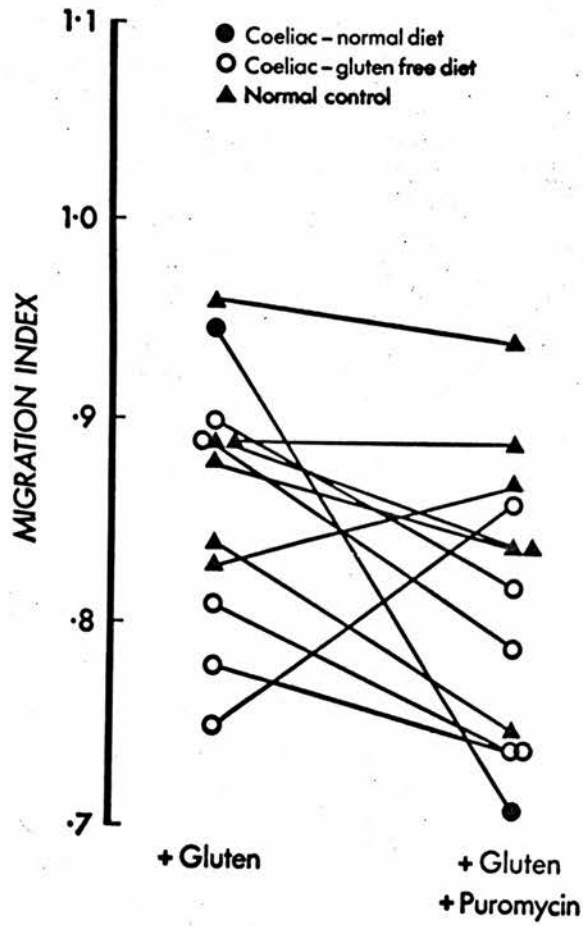


FIG. 26. Migration indices to gluten fraction III in 6 normal controls, one untreated coeliac and 5 treated coeliacs with and without the addition of puromycin at $15\mu\text{g/ml}$ to the culture medium

LMI test using subpopulations of leucocytes1) Using PPD as antigen

Figure 27 illustrates the results of the LMI test performed in the standard manner and with purified polymorphonuclear leucocytes, purified mononuclear cells and purified polymorphs with added T lymphocytes and with added B lymphocytes, using PPD as the antigen in three Mantoux-positive and one Mantoux-negative subjects. Significant migration inhibition of cells from the Mantoux-positive subjects is seen only in the standard test and in the test using polymorphs with added T cells. Polymorphs alone, mononuclear cells alone and polymorphs with B cells show no inhibition.

2) Using gluten fraction III

Figure 28 shows migration indices to gluten fraction III for eight controls, five untreated coeliacs and five treated coeliacs using the standard LMI test and the test performed with the same cell types as in Figure 27. With the standard test the coeliac patients show a significant reduction of migration index compared with controls (mean migration index for coeliacs 0.84, mean for controls 0.91, $p < 0.05$ by Wilcoxon's rank sum test). However, a similar difference is seen when the test is performed with polymorphs only (mean for coeliacs 0.86, controls 0.99,

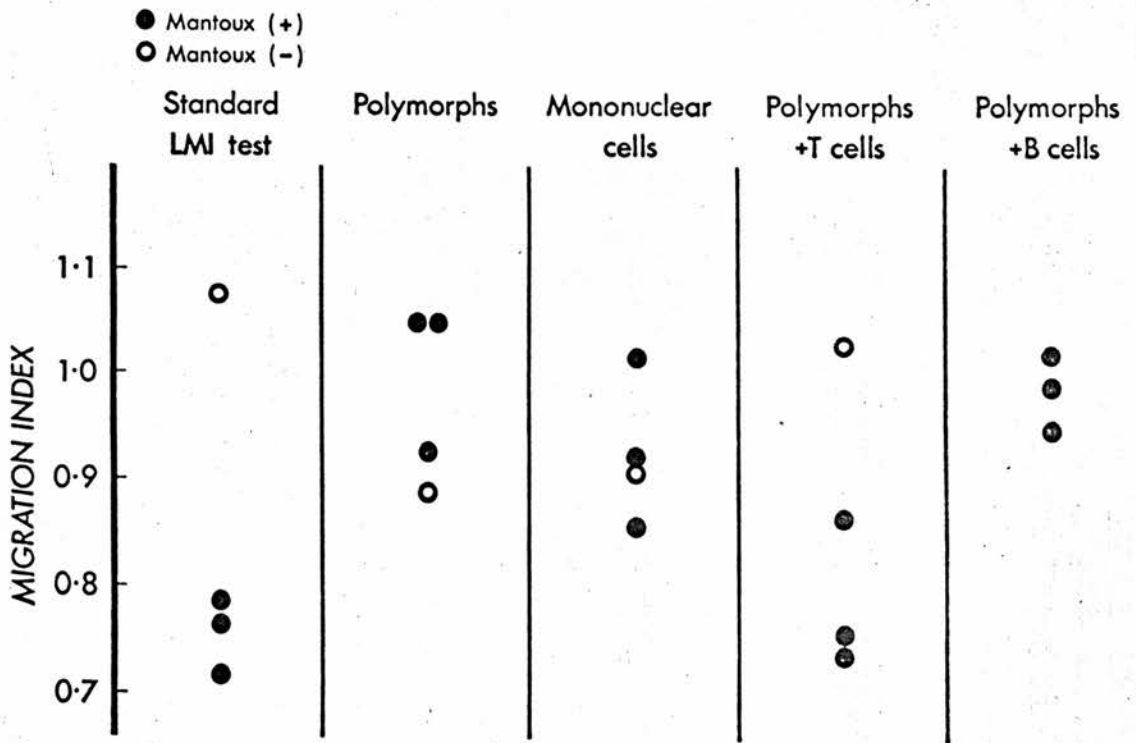


FIG. 27. Migration indices to PPD in 3 Mantoux positive and one Mantoux negative individuals using the standard LMI test performed with subpopulations of leucocytes

$p < 0.05$). Mononuclear cells alone were not inhibited either in coeliacs or controls (means: coeliacs 0.96, controls 0.96). When the test was performed with polymorphs with added T cells results were similar to those with polymorphs alone (means: coeliacs 0.89, controls 0.97) and this also held with results using polymorphs with added B cells (means: coeliacs 0.91, controls 0.94) though the difference between controls and coeliacs fails to reach statistical significance using these cell combinations.

Figure 29 shows the correlation between individual migration indices to gluten fraction III using the standard LMI test and that performed with purified polymorphs. There is a significant correlation between the results of the two tests using both parametric and non-parametric tests ($r = 0.62$, $t = 3.2$, $p < 0.01$; Spearman's rank correlation coefficient = 0.64, $p < 0.01$). Furthermore, the slope of the line of best fit is 0.91 with a standard deviation of the slope of 0.28. The slope does not, therefore, differ significantly from unity ($t = 0.33$) nor does the intercept differ significantly from zero (y intercept = 0.13, SD = 0.24, $t = 0.53$).

Figure 30 shows a similar correlation between the standard LMI test and that performed with polymorphs

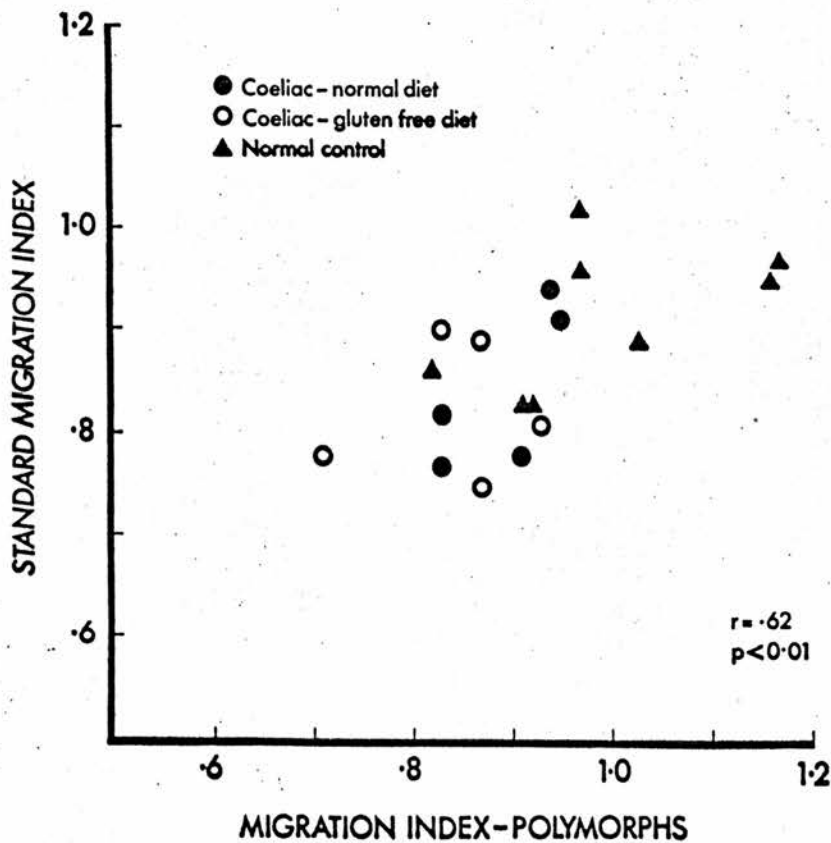


FIG. 29. Correlation between migration indices with gluten fraction III as antigen in controls and coeliacs using the standard LMI test and the LMI test performed with purified polymorphs alone

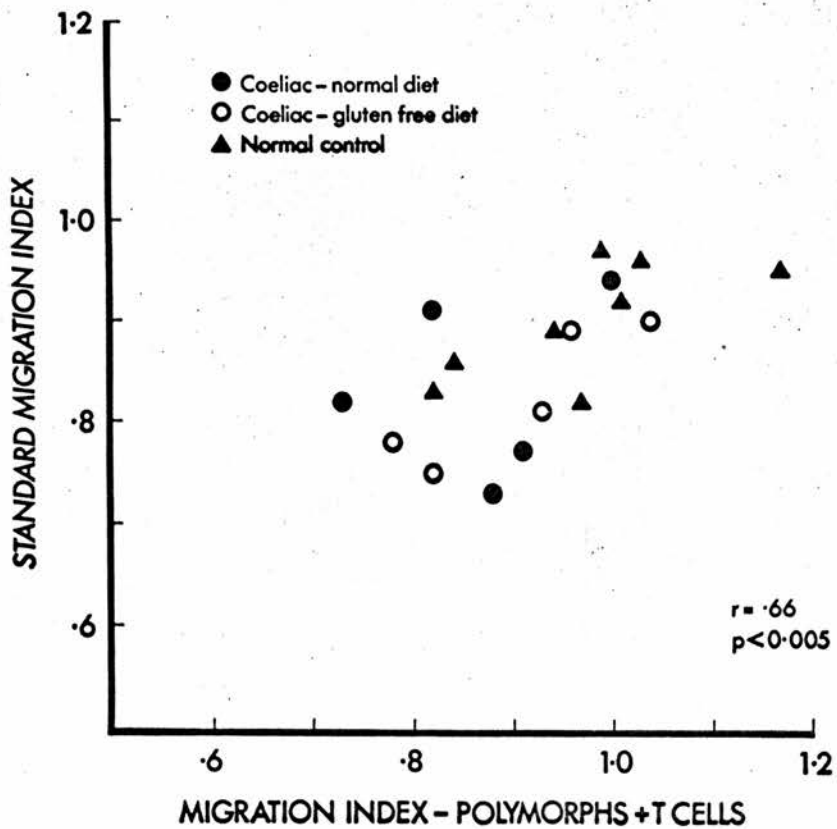


FIG. 30. Correlation between migration indices with gluten fraction III as antigen in controls and coeliacs using the standard LMI test and the LMI test performed with polymorphs with added T cells

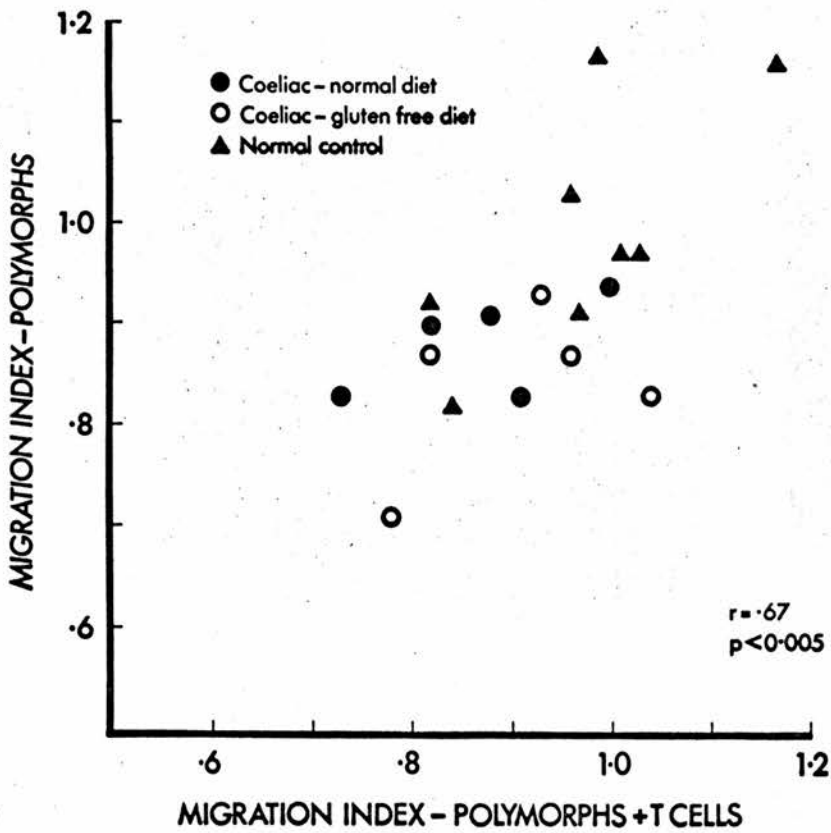


FIG. 31. Correlation between migration indices with gluten fraction III as antigen in controls and coeliacs using the LMI test performed with purified polymorphs and with polymorphs with added T cells

with added T cells. There is again a significant correlation between results ($r = 0.66$, $p < 0.005$). Figure 31 shows the correlation between results of the tests performed with polymorphs alone and with polymorphs with added T cells. This too is significant ($r = 0.67$, $p < 0.005$). There are similar positive correlations between results of the test performed with polymorphs with the test performed with polymorphs plus B cells ($r = 0.74$, $p < 0.005$) and between the test with polymorphs plus T cells and that with polymorphs plus B cells ($r = 0.65$, $p < 0.01$). There is thus no apparent effect on the results of the test by incorporation of T cells or B cells into the system.

Effect of serum incubation on LMI test

The effect of incubation with sera from six coeliac patients on the migration index of leucocytes from a single healthy control is shown in Figure 32. Cells from the control subject consistently gave a migration index with gluten fraction III of around 0.9 (mean of 4 experiments 0.91, SD 0.02). After incubation with coeliac sera and washing, however, the cells were markedly inhibited by gluten fraction III (mean migration index 0.75, SD 0.05). This sensitisation to gluten was prevented by incubation with aggregated human IgG before incubation with the coeliac sera (mean migration index 0.89, SD 0.05).

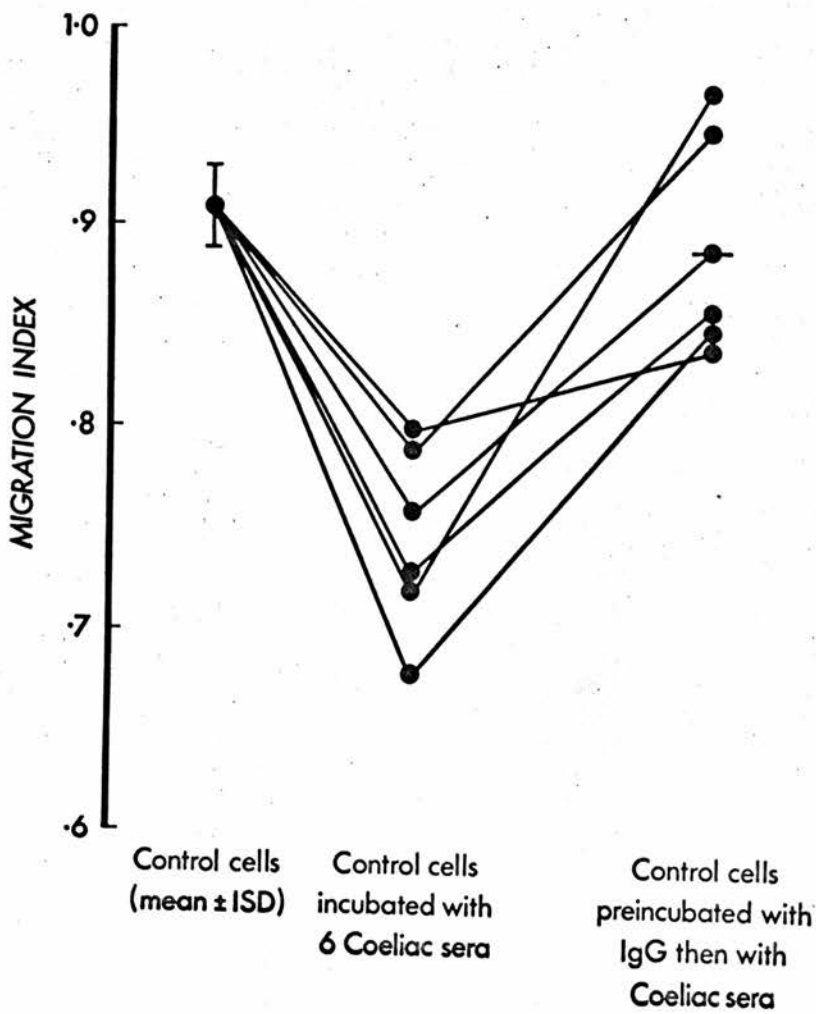


FIG. 32. Effect of incubation with coeliac sera on migration index of normal leucocytes with gluten fraction III as antigen and effect of pre-incubation with aggregated human IgG

Leucocyte inhibitory factor production by jejunal mucosa

The correlation between results of the direct LMI test using peripheral blood leucocytes and of the indirect LMI test to assess production of leucocyte inhibitory factors by cultured jejunal mucosa in 16 coeliac patients is shown in Figure 33. There is a significant inverse correlation between blood and biopsy results ($r = 0.56$, $t = 2.58$, $p < 0.025$); that is, when there is marked production of inhibitory factors by the jejunal biopsy, blood leucocytes do not show such inhibition; when there is little inhibition in the indirect test using the biopsy, blood leucocytes are likely to be inhibited in the direct test. Figure 34 shows the results of the two tests performed on a single coeliac patient, both untreated and after eight months on a gluten-free diet. Initially the direct test shows little inhibition (migration index 0.92) whereas there is evidence of inhibitory factor production by jejunal mucosa (indirect migration index 0.77). After eight months treatment the position is reversed, direct migration index falling to 0.79 whilst the migration index from the indirect test has risen to 1.05.

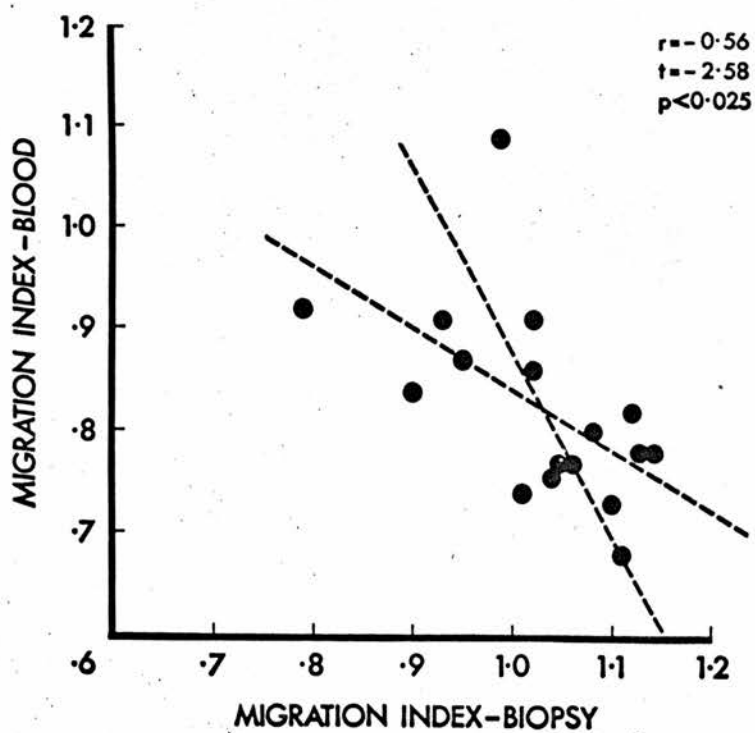


FIG. 33. Correlation (with lines of best fit) between the direct LMI test with blood leucocytes and the indirect LMI test with cultured jejunal biopsies in 16 coeliacs with gluten fraction III as antigen

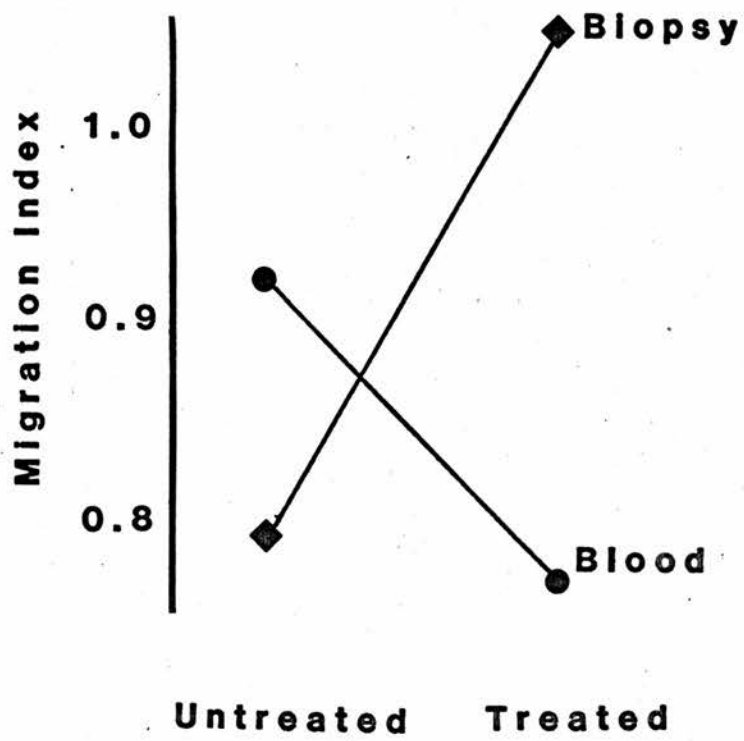


FIG. 34. Migration indices to gluten fraction III for the direct LMI test with blood leucocytes and the indirect test with cultured jejunal biopsies in a single coeliac patient studied untreated and after 8 months of gluten exclusion

Jejunal biopsy and lymphocyte co-culture

Histology

Full results of quantitative histological assessment of the biopsies pre-culture and after culture in control medium, medium with added gluten fraction III, added lymphocytes and added gluten with added lymphocytes are given in Table 4 of the Appendix.

Figure 35 illustrates the enterocyte heights of biopsies from coeliac patients before culture and after 24hr culture in control medium. Mean enterocyte height before culture was 29.1μ and after culture 25.4μ . This difference is significant by both Wilcoxon's rank sum test ($p < 0.01$) and Student's t test ($p < 0.001$). (Appendix Table 4 gives statistical significance results using the t test; Figures in the text give results from Wilcoxon's test).

Figure 36 shows results comparing biopsies cultured in control medium with those cultured with gluten fraction III alone and with lymphocytes alone. Mean enterocyte height of biopsies cultured with lymphocytes was 24.6μ (3.1% lower than that of biopsies cultured in control medium). Mean enterocyte height of biopsies cultured with gluten fraction III was 24.7μ (2.8% lower than biopsies cultured in control medium). Neither of these differences is statistically

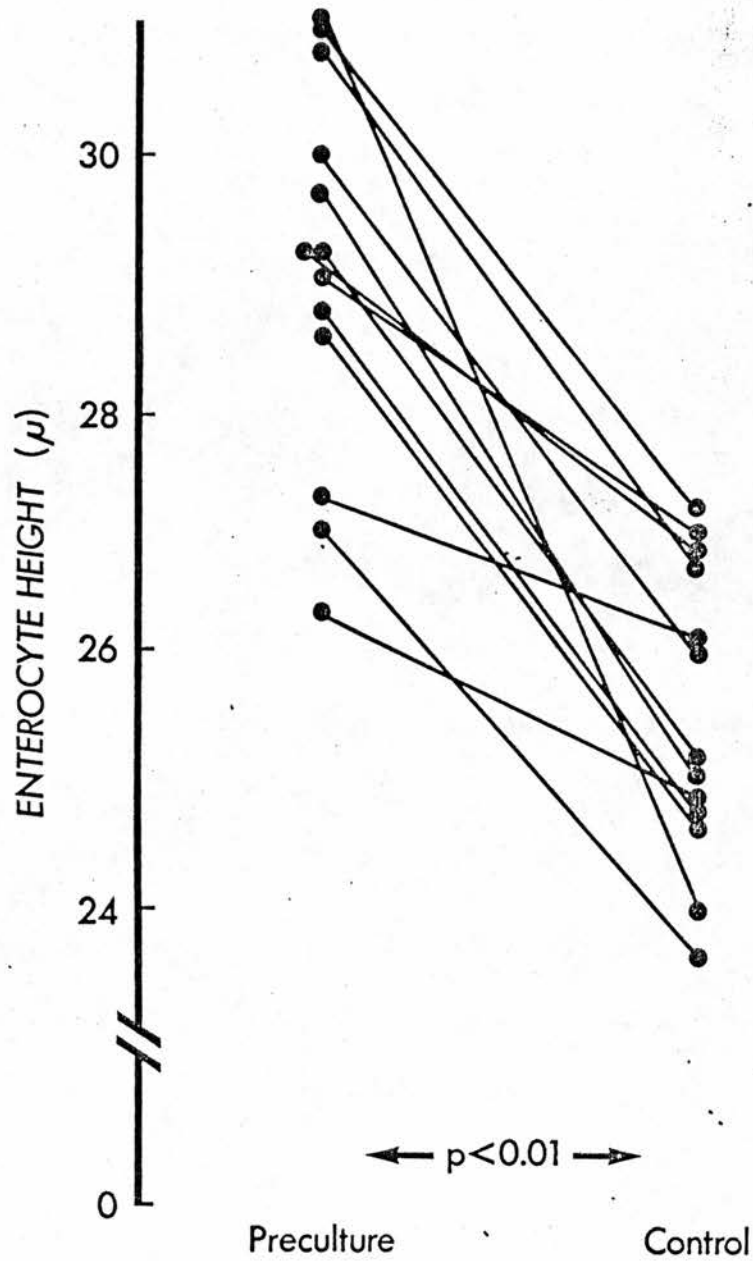
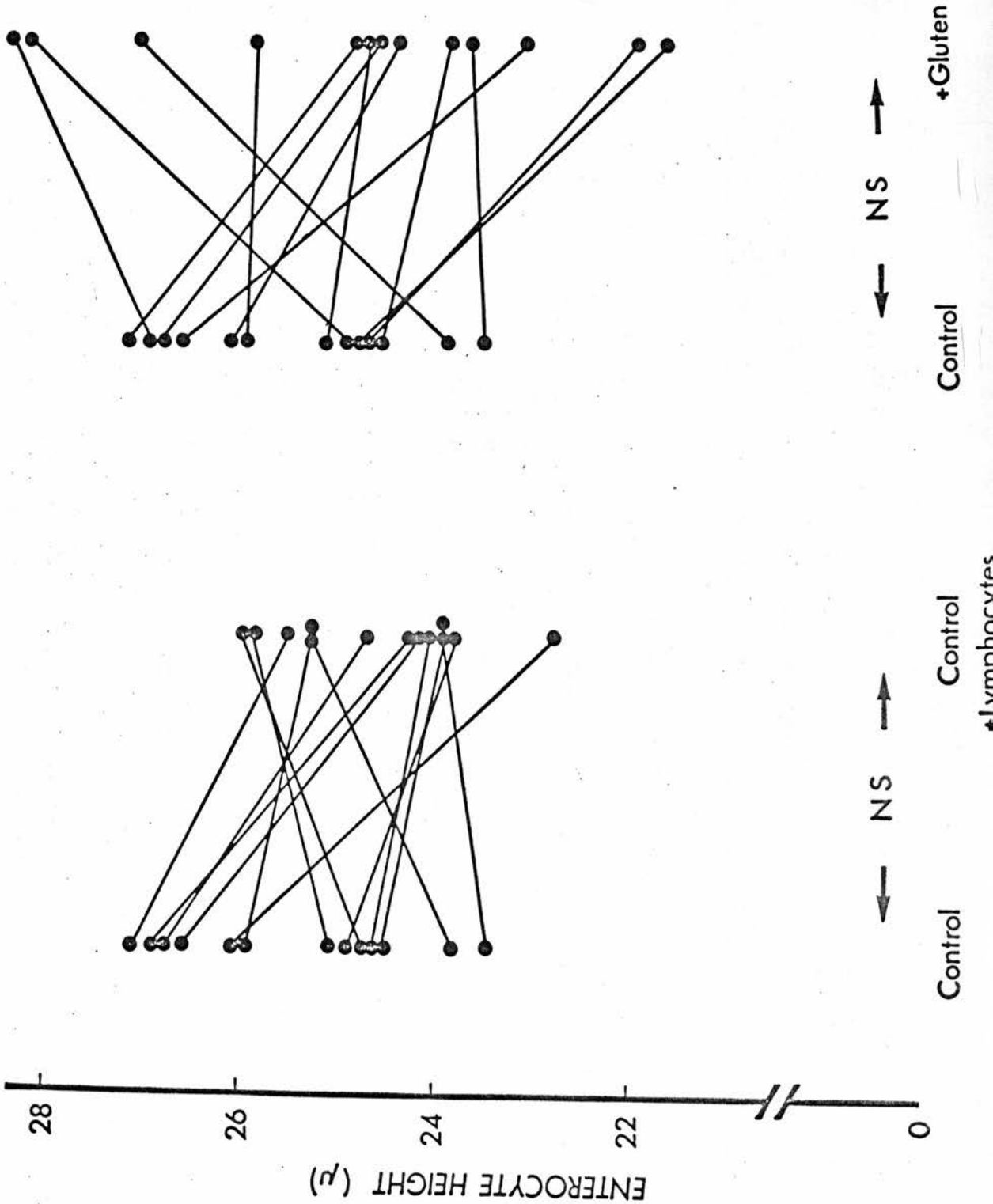


FIG. 35. Enterocyte heights of biopsies from coeliac patients before and after 24 hour culture in control medium

FIG. 36. Enterocyte heights of biopsies from coeliac patients after 24 hour culture in control medium compared with enterocyte heights after culture in medium with added lymphocytes alone and in medium with added gluten alone



significant by either statistical test. Biopsies cultured with added gluten and with added lymphocytes had a mean enterocyte height of 23.1μ (Figure 37). This represents a 9.1% reduction compared with biopsies cultured in control medium and is significant ($p < 0.01$ by Wilcoxon's rank sum test, $p < 0.001$ by Student's t test). Figure 38 compares biopsies cultured in the presence of both gluten and lymphocytes with biopsies cultured with added gluten alone and with added lymphocytes alone. Mean enterocyte height of biopsies cultured with gluten and lymphocytes was significantly lower than both that of those cultured with gluten alone ($p < 0.01$ by Wilcoxon's test, $p < 0.02$ by Student's t test) and those cultured with lymphocytes alone ($p < 0.01$ by Wilcoxon's test, $p < 0.005$ by Student's t test).

The non-coeliac controls showed a similar reduction in enterocyte height after culture in control medium (mean 27.6μ) compared with preculture values (mean 30.75μ) as did the coeliac patients. However, there were no significant differences in enterocyte height between biopsies from normal controls cultured in control medium, with gluten, with lymphocytes and with gluten and lymphocytes (Figure 39).

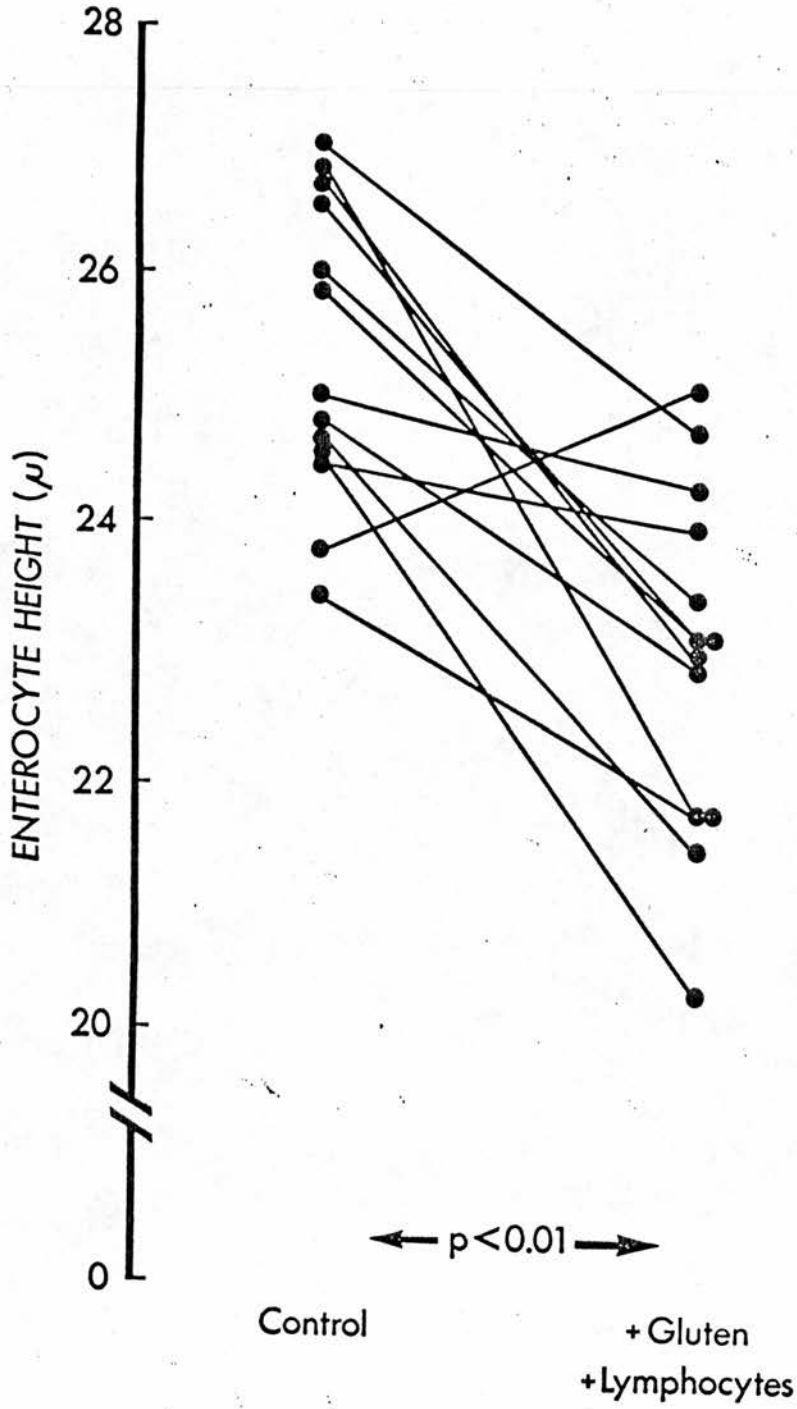
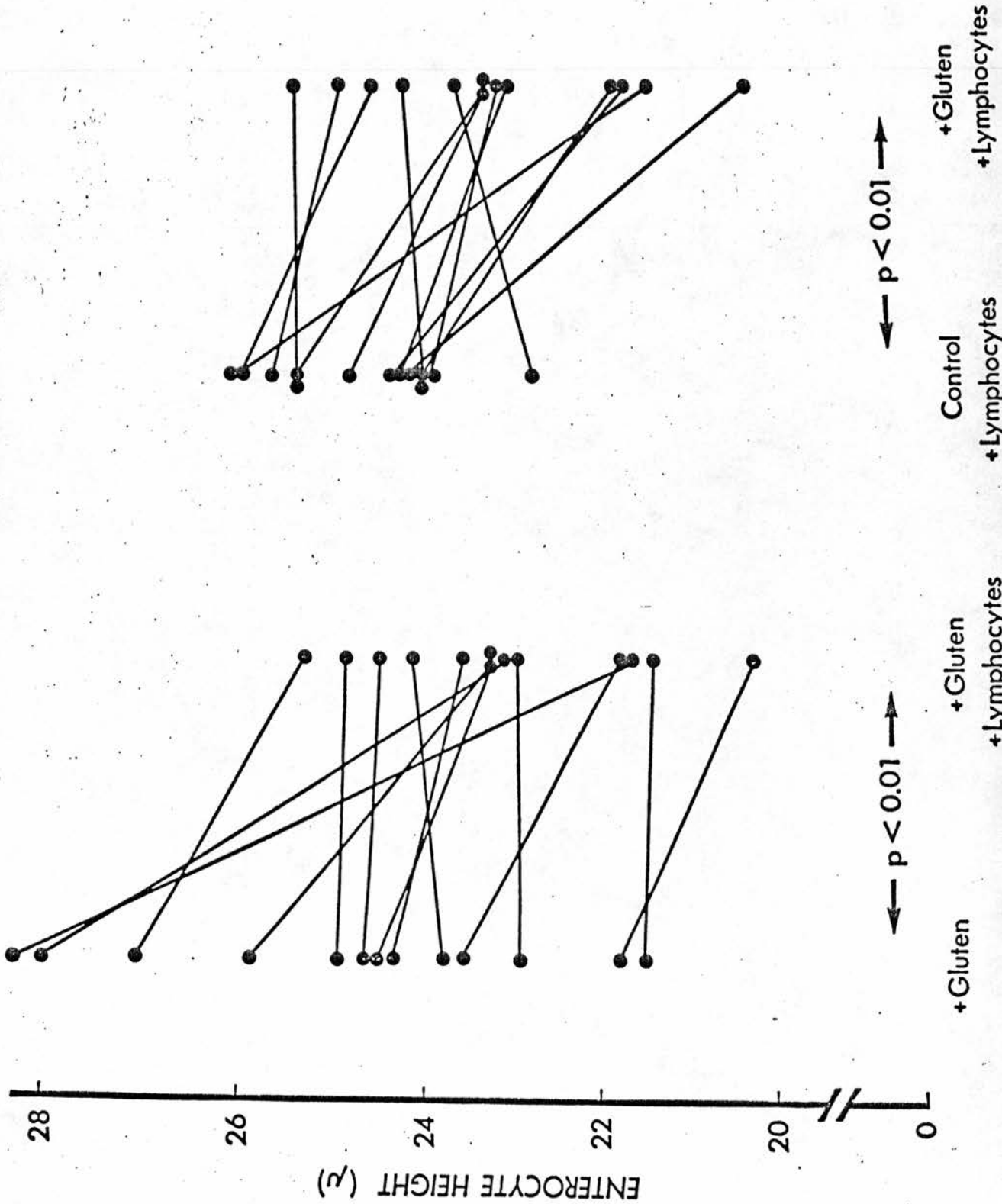


FIG. 38. Enterocyte heights of biopsies from coeliac patients cultured in medium with added gluten and added lymphocytes compared with enterocyte heights when cultured with added gluten alone or with added lymphocytes alone



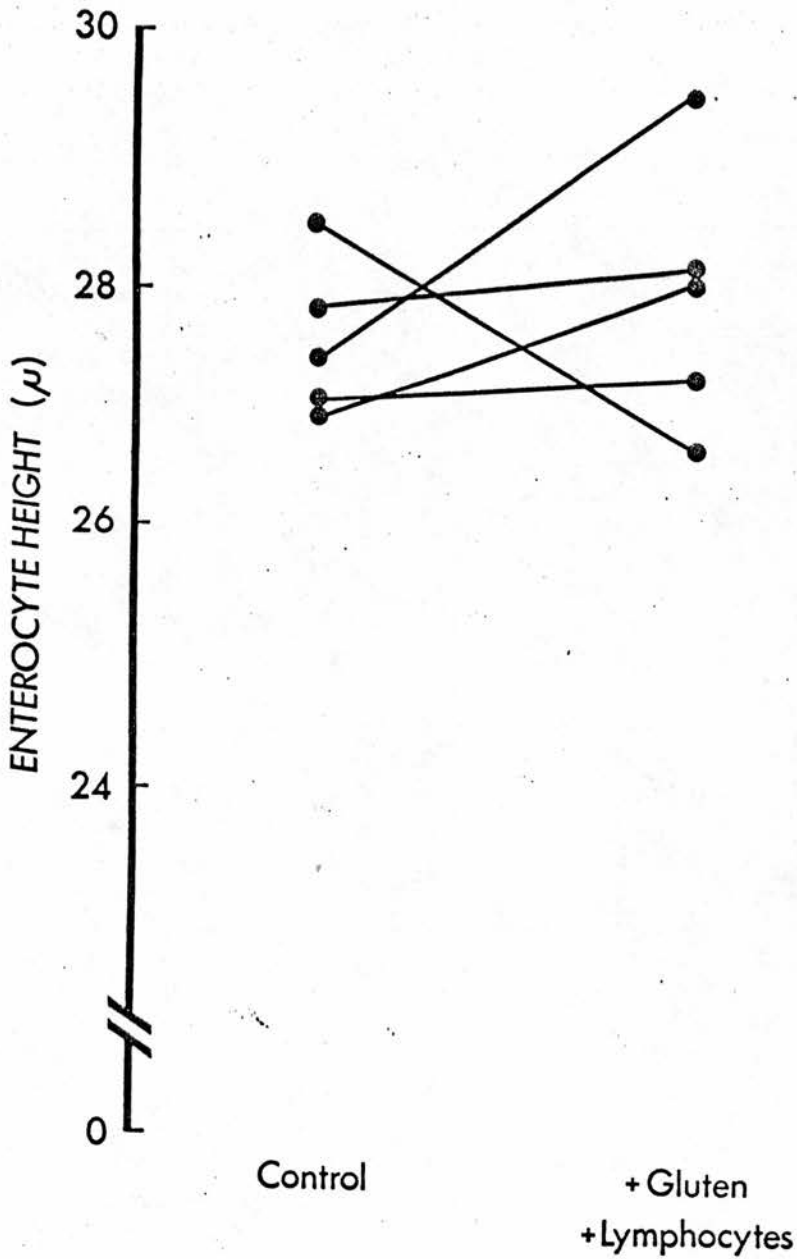


FIG. 39. Enterocyte heights or biopsies from control subjects after 24 hour culture in control medium and after culture in medium with added gluten and added lymphocytes

Figure 40 illustrates a section from a biopsy from a treated coeliac patient after 24hrs culture in control medium. The biopsy is well preserved and the epithelial cell layer is regular. Figure 41 shows a section from a biopsy from the same patient cultured for 24hrs in the presence of gluten fraction III and autologous lymphocytes. There is obvious morphological deterioration and, in particular, the epithelial cells are disorganised, irregular and of reduced height. These changes are more clearly seen in the higher power views in Figures 42 and 43 which also show typical enterocytes chosen for measurement of epithelial cell height. Figure 44 shows a biopsy from a normal control cultured with gluten and lymphocytes. In contrast to Figure 41, this biopsy is well preserved and the epithelial cells remain normal.

Leucocyte migration indices for the 13 coeliac patients are shown in Table 10, together with the differences in mean enterocyte height between specimens cultured in control medium and those cultured with gluten and lymphocytes and the differences between specimens cultured with gluten alone and those cultured with gluten and lymphocytes. There is no correlation between migration index and differences in enterocyte height either for biopsies cultured in control medium



FIG. 40. Biopsy from treated coeliac patient after 24 hour culture in control medium (Haematoxylin & eosin. x 100)

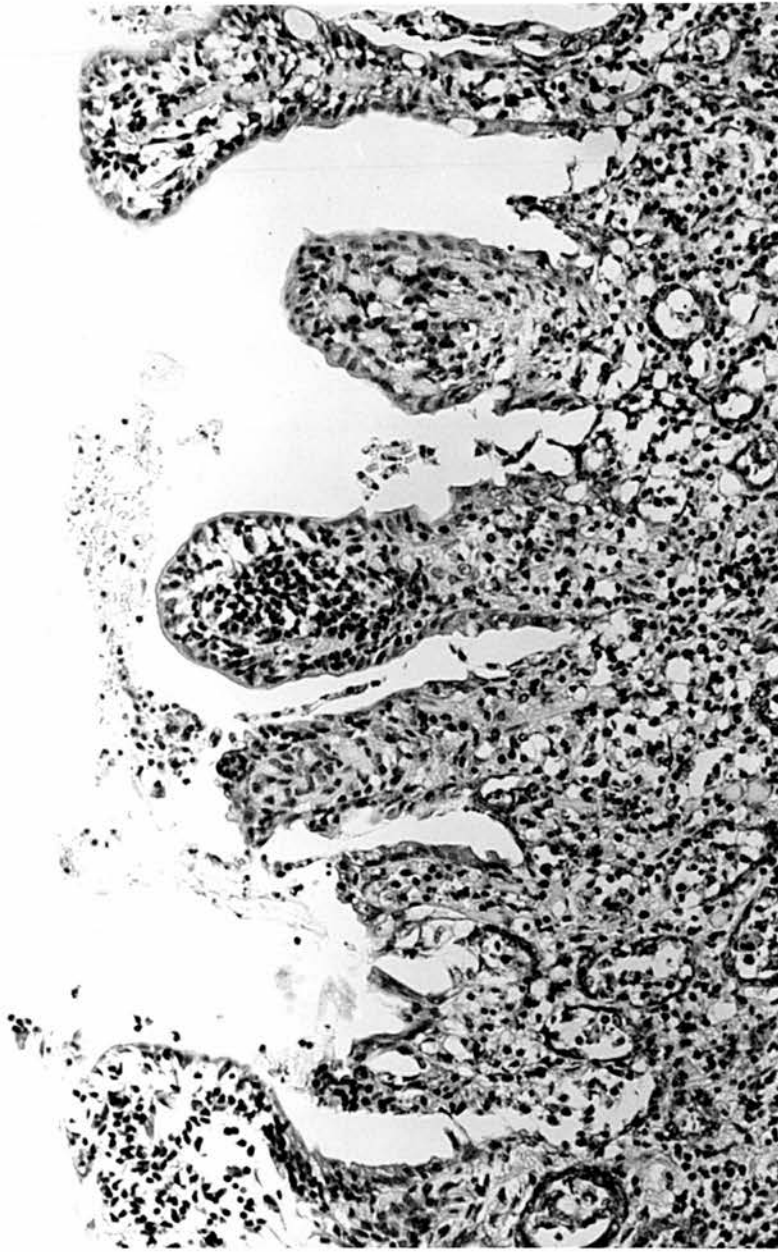


FIG. 41. Biopsy from treated coeliac patient after 24 hour culture in medium with added gluten and with added lymphocytes (Haematoxylin & eosin. x 100)



FIG. 42. Biopsy from treated coeliac patient after 24 hour culture in control medium. Arrow indicates typical enterocyte measured (Haematoxylin & eosin. x 250)

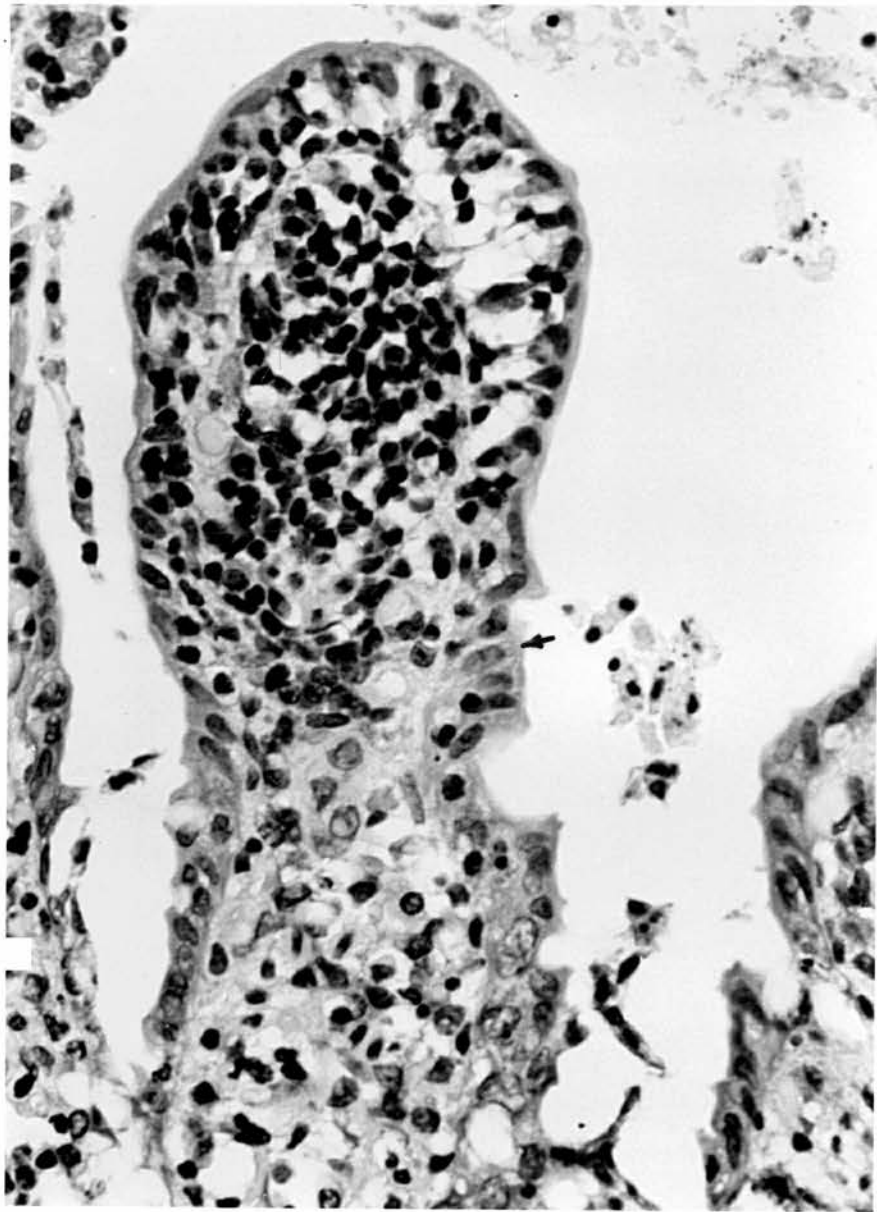


FIG. 43. Biopsy from same patient as in Figure 42 after 24 hour culture in medium with added gluten and with added lymphocytes. Arrow indicates typical enterocyte measured (Haematoxylin & eosin. x 250)



FIG. 44. Biopsy from normal control after 24 hour culture in medium with added gluten and added lymphocytes (Haematoxylin & eosin. x 100)

TABLE 10

Leucocyte migration indices to gluten fraction III antigen in treated coeliac biopsies and differences in enterocyte height between jejunal biopsies cultured in control medium and those cultured with gluten and lymphocytes ((C)-(+G+L)) and of biopsies cultured with gluten alone and those cultured with gluten and lymphocytes ((+G)-(+G+L))

PATIENT	LEUCOCYTE MIGRATION INDEX	ENTEROCYTE HEIGHT CHANGES (μ)	
		(C)-(+G+L)	(+G)-(+G+L)
1	0.74	+2.55	+0.82
2	0.71	+1.70	+1.75
3	0.92	+0.70	+0.20
4	0.80	-1.35	+1.80
5	0.91	+0.50	-0.30
6	0.91	+3.56	+1.31
7	0.68	+1.85	+5.10
8	0.91	+5.15	+6.60
9	0.76	+3.25	+0.05
10	0.77	+2.75	+2.60
11	0.87	+3.60	0
12	0.86	+2.25	-0.10
13	0.89	+4.24	+1.44

compared with those cultured with gluten and lymphocytes (Kendall's rank correlation $t = 0.22$, $p > 0.1$) or for biopsies cultured in gluten-containing medium compared with those cultured with gluten and lymphocytes ($t = 0.29$, $p > 0.1$).

Alkaline phosphatase

Full results of alkaline phosphatase assays on the cultured jejunal biopsies are given in the Appendix Tables 5-7 with results expressed in relation to the protein content, wet weight and DNA content of the biopsies. Mean alkaline phosphatase activity in the pre-culture biopsies was 38.3mU/mg.protein (SD 20.8). After culture for 24hrs this rose to 59.9 mU/mg.protein (SD 27.9) for specimens cultured in control medium, to 64.2mU/mg.protein (SD 36.9) for specimens cultured with lymphocytes, to 59.8mU/mg. protein (SD 37.9) for specimens cultured with gluten alone and to 55.0mU/mg.protein (SD 28.6) for specimens cultured with gluten and lymphocytes. There were no significant differences between any of the post-culture values and this also applies to the results when expressed in relation to wet weight of biopsy or to DNA content of the biopsy.

Lymphocyte viabilities

Viability of lymphocytes after 24hr culture with jejunal biopsies was always over 90%.

Phosphatidyl Inositol Turnover

Results of phosphatidyl inositol turnover as assessed by uptake of labelled myoinositol by jejunal biopsies cultured in the presence and absence of gluten fraction III for healthy controls, untreated coeliacs and treated coeliacs are shown in Figure 45, with the turnover expressed as disintegrations per minute per mg of protein. Mean value for healthy controls in the absence of gluten was 68.1×10^3 dpm/mg.protein and in the presence of gluten 74.5×10^3 dpm.mg.protein. For biopsies from untreated coeliacs the mean values were 83.7×10^3 dpm/mg.protein in control medium and 81.7×10^3 dpm/mg.protein in gluten-containing medium. The treated coeliacs gave results of 79.0×10^3 dpm/mg.protein in control medium and 82.2×10^3 dpm/mg.protein in gluten-containing medium. Results for the same groups expressed as an index (calculated as radioactive uptake in the presence of gluten divided by uptake in control medium) are shown in Figure 46.

There are no differences between the diagnostic groups either in absolute levels of radioactive uptake nor in the effect of gluten on the uptake. There is, in fact, no consistent effect of gluten in any group.

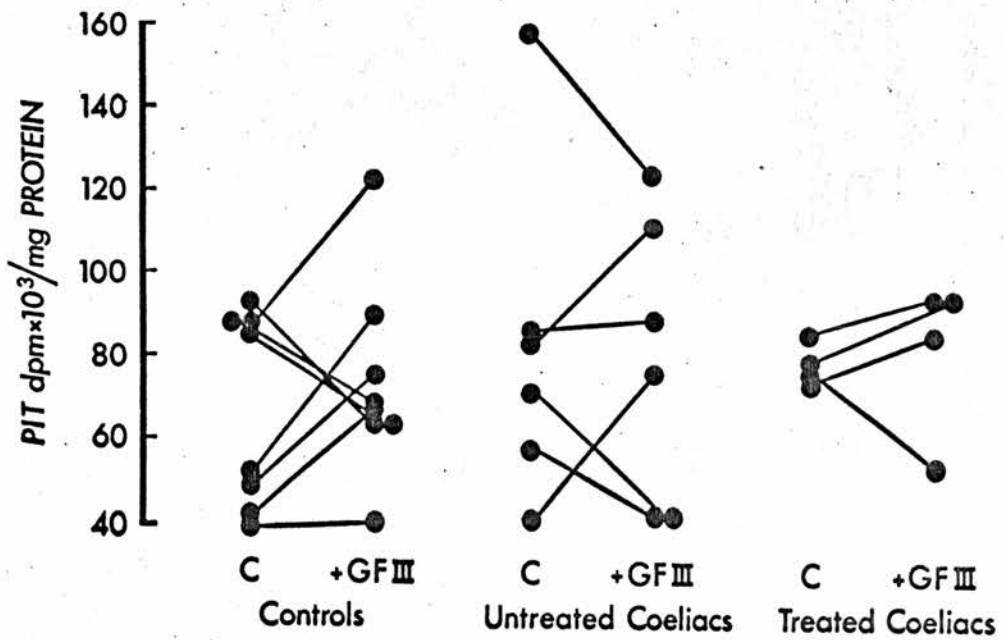


FIG. 45. Phosphatidyl inositol turnovers for jejunal biopsies cultured with and without gluten fraction III in controls, untreated coeliacs and treated coeliacs expressed as disintegrations per minute per mg. of protein

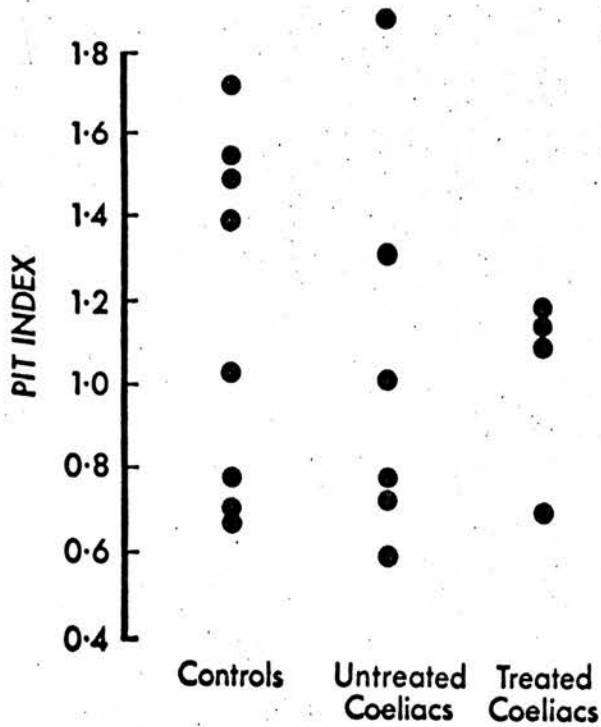


FIG. 46. Phosphatidyl inositol turnovers for jejunal biopsies from controls, untreated coeliacs and treated coeliacs expressed as a ratio of radioactive uptake in the presence of gluten to uptake in control medium (PIT index)

D I S C U S S I O N

"Possibly the result may have been brought about by
the natural obstinacy of all things in this world"

Three Men in a Boat
Jerome K. Jerome, 1889.

The LMI test

A large part of this thesis is concerned with the use and interpretation of the leucocyte migration inhibition test in coeliac disease. A considerable effort was therefore expended in making sure that the test was reproducible and that factors known to influence results factitiously were excluded (Maini et al. 1973). From the results shown in Figure 7 and Table 4, it can be seen that this was successful. In the absence of dietary changes known to influence results the test is shown to give consistent results when individuals are tested repeatedly and it was possible to calculate the magnitude of the change in migration index in this system which is statistically unlikely to be due to chance at the 5% level. This is calculated at just under 0.1. Whatever the test is measuring it is at least measuring it consistently. These results conflict with those reported by the Glasgow group (Filer et al. 1981; Pattison et al. 1981) who studied both agarose microdroplet and capillary tube methods of performing the LMI test and concluded that at least 30 replicates were required to have confidence that mean migration area of a set of replicate cultures was within 5% of the true mean for either system. Much of the argument used by the

Glasgow group is, however, based on computer simulation of experimental results and is concerned with areas of migration rather than migration indices. When the latter are discussed the emphasis is on the statistical validity of a single result, whereas in this study almost all of the conclusions are drawn from comparisons between groups of patients. Furthermore, the variability of the migration areas which is quoted by the Glasgow workers for the capillary tube method (coefficient of variation from 7.7% to 28.8% calculated on 15-26 replicates) seems large compared with the 0.1 - 16.4% (on quadruplicate cultures) in this study, perhaps suggesting that not enough attention was paid to the technical details discussed in the Methods section.

The dose-response to gluten fraction III and time-course experiments in this study gave results very similar to previous workers (Bullen & Losowsky, 1978b; Haeney & Asquith, 1978) and at the concentrations chosen for study no antigen showed significant toxicity nor any tendency for selective toxicity to coeliac lymphocytes. The criticism that results of any migration inhibition test may be due to "non-specific" toxicity affecting cell motility is, of course, unanswerable as "non-specific" inhibition in the

patient group cannot be assessed. However, the changes in migration inhibition produced by the various manipulations such as pre-incubation with coeliac serum (which sensitises normal leucocytes to gluten but does not affect their migration in control medium) and the blocking of this effect by IgG does suggest that such an explanation is unlikely and that the test is indeed reflecting some immunological phenomenon.

Leaving aside for the moment the results pertaining to the mechanism by which gluten affects the migration of leucocytes from coeliac patients (i.e. precisely what immunological phenomenon is being measured by the test) what of the other problems outlined in the Introduction relating to the interpretation of LMI test results in coeliac disease? First, what of the diagnostic power of the test? The results presented here are in line with most of the published work on the direct LMI test - that is they show that even if HLA status is ignored, the test does not separate coeliac and non-coeliac groups which, though they differ statistically, overlap to such an extent that the test is unlikely to aid diagnosis in an individual case. In this regard, it is important to note that the differences between controls and coeliacs are less when the coeliacs are taking a normal (gluten-

containing) diet, which is, of course, the case when a diagnostic problem in an individual case occurs. The suggestion that suspected coeliac patients should be given a gluten-free diet for some months to increase the power of the test (Ashkenazi et al. 1981) cannot be taken seriously as, apart from the unwieldiness of such a diagnostic procedure, no data exist on the effect of such a diet on the LMI test results of normal individuals.

Also of relevance to the diagnostic power of the LMI test are the experiments relating LMI results to HLA status and those comparing the B2 and fraction III subfractions of gluten. Ashkenazi et al. (1980) suggest that B2 gives superior results to gluten fraction III in their agar-drop system. In a direct comparison between B2 and gluten fraction III (see Figures 21 and 22) no correlation between results with the two antigens was shown and in fact B2 produced no significant inhibition of migration in any patient studied. This is perhaps unsurprising as the concentration of B2 used was almost certainly sub-optimal for the capillary tube LMI test and dose-response studies were precluded by the limited quantity of material available. Nevertheless, Ashkenazi et al. (1980) compared the optimal dose of B2 (established by

dose-response studies) with a single arbitrary dose of gluten fraction III in a direct agarose drop system to reach their conclusion that B2 was superior. Perhaps the only fair conclusion to be reached from the present results is that comparisons between different antigenic materials in any LMI system are unjustified unless full dose-response and time-course experiments are included so that optimal responses can be compared.

Of greater importance and interest are the observations on the effects of HLA status on results of the LMI test. One possible explanation for the high prevalence of the HLA-B8 antigen in coeliac disease (80% as against 20% in the general population (Falchuk et al. 1972)) is that possession of HLA-B8 is associated with an increased immune response to gluten. As has been discussed, there is strong evidence for specific immune response genes to a variety of antigens in animals (including an H-2 linked response to gluten in the mouse (Johnson et al. 1980)). In man the position is less clear as the antigens used are in general complex and presumably bear many antigenic determinants, unlike the synthetic antigens used in most animal studies. However, HLA status has been shown to be associated with altered immune reactivity to a variety of antigens and genetic

analysis in one study has indicated that a single gene is involved despite the complex nature of the antigen (Sasazuki et al. 1980).

In the present study, a clear increase in immunity to gluten (as measured by the LMI test) is shown in normal controls with HLA-B8 compared with those lacking HLA-B8. This finding is consistent with the hypothesis that there may be a single immune response gene for gluten in linkage disequilibrium with HLA-B8 but does not provide proof. Further studies, including large family studies with genetic analysis such as that performed by Sasazuki et al. (1980) are required to further investigate this hypothesis.

The possible importance of the existence of such an immune response (Ir) gene is seen when the results of the coeliac patients are examined. Here there is no difference in response between HLA-B8 coeliacs and non-HLA-B8 coeliacs, and both show increased response to gluten compared with non-HLA-B8 controls. This implies that for coeliac disease to develop it is necessary to have the putative Ir gene for gluten. However, many normal controls having HLA-B8 presumably also possess this putative gene which is thus not sufficient for the development of coeliac disease (though the observations of Doherty & Barry (1981) on

gluten-induced small bowel changes in relatives of coeliac disease patients may be relevant here). This position is similar to that seen in HLA-linked immune responses to ragweed pollen and the development of clinical hay-fever (Levine et al. 1972). It is also of interest that earlier studies of anti-gluten antibodies (Scott et al. 1974) showed a similar pattern, with increased titres in normals and non-coeliac disease patients with HLA-B8 but no effect of HLA-B8 in coeliac disease.

The influence of HLA-B8 on results of the LMI test also has important implications when considering the possible diagnostic use of the test. Current enthusiasts for the LMI test as a diagnostic aid (Ashkenazi et al. 1978; Ashkenazi et al. 1981; O'Farrelly et al. 1982) have presented data from the agarose drop method and the indirect LMI test comparing coeliacs with normal controls. If HLA-B8 has an effect on results of these assays then the demonstrated differences may merely reflect the increased prevalence of HLA-B8 in a coeliac population. Before these tests can be accepted, it is thus necessary to re-evaluate results with reference to the HLA status of the control groups.

HLA-DR3 is more closely associated with coeliac disease than HLA-B8 and it is thus rather disappointing that no significant increase in immune response to gluten was shown in controls having this antigen. This may merely reflect the smaller numbers studied.

The effect of gluten-exclusion on increasing detectable immune reactivity to gluten of circulating leucocytes in coeliac disease has been known for some years (Holmes et al., 1976; Bullen & Losowsky, 1978b). Results from this study agree with earlier work and have since been confirmed by work using the agarose drop LMI test (Ashkenazi et al. 1981). The initial fall in migration index (in the arbitrarily-defined early treated group) is consistent with the release of committed gluten-reactive lymphocytes from the gut proposed by Holmes et al. (1976). Antigen-specific trapping of lymphocytes in the gut has been demonstrated in rats sensitised to cholera toxoid (Pierce & Gowans, 1975). The later decline in immunity has not previously been demonstrated and would suggest a gradual reduction in the population of gluten-reactive cells after prolonged lack of antigenic stimulation.

The demonstration of the inverse relationship between production of inhibitors of leucocyte migration

by jejunal mucosa and in peripheral blood is also in accord with the proposed shift of gluten-reactive cells with changes in the diet. This inverse relationship has not previously been directly demonstrated. As will be discussed later, other explanations for the phenomenon are possible.

One of the major problems of any immunological investigation into a disease lies in deciding whether any positive findings may be pathogenetically important or are merely epiphenomena. As was mentioned in the Introduction, early interest in the pathogenic role of anti-gluten antibodies waned with the realisation that non-coeliacs sometimes possessed this antibody and that coeliacs often produced antibodies to a wide range of dietary antigens. This last observation has been explained by postulating "leakiness" of the damaged mucosa in coeliacs with consequent increased exposure of the immune system to dietary antigens (Ferguson & Carswell, 1972; Asquith & Haeney, 1979). The specificity of the LMI test with regard to antigen has not previously been studied with a range of dietary antigens.

The food antigens used in this study were chosen in order to try and maximise the chances of obtaining a positive result. Thus, antibodies to all antigens

chosen are relatively common in coeliac disease (Kivel et al., 1964; Kumar et al., 1976; Ferguson & Carswell, 1972). Crude preparations of these foods were used as similar preparations are usually used in antibody studies and seem more likely to include the actual antigens encountered in the diet than more purified and homogeneous protein extracts. The use of bovine serum albumin posed some problems because of potential cross-reaction with the foetal calf serum used in the culture medium. However, it was included in preliminary studies as it has been found that there is a considerably higher prevalence of antibody to BSA than to foetal calf serum in coeliac disease (Kumar et al., 1976). This is presumably due to reactions to polymers found in commercially prepared BSA (Solli & Bertolini, 1977) similar to those found in cooked meat in the diet. It is known that polymerised or heated BSA differs antigenically from native monomeric BSA (Strachambova-McBride et al. 1980).

The observation that food antigens other than gluten give similar results to gluten in the LMI test weakens the argument that the test is measuring an immune phenomenon which is important in pathogenesis. It does not, of course, disprove the hypothesis as positive results to the other antigens may be epiphenomena whilst those to gluten may be important.

The degree of immunity to egg and BSA shown by the LMI test seems to correlate with the degree of mucosal abnormality, decreasing as the mucosa improves. This is similar to results of earlier antibody studies (Kumar et al.,1976) and supports the concept of "leakiness" of the mucosa as an explanation for the findings. The different pattern of response to gluten after starting treatment, with an early increase in immunity both in antibody studies (Rossipal, 1974; Scott, 1975; Stern et al.,1979a) and using the LMI test, could suggest that gluten does differ from the other antigens and that these immune reactions may be pathogenetically important. This argument, however, ignores the fact that the treatment is exclusion of gluten from the diet and not exclusion of these other antigens. The vital experiments of assessing the response of normal controls to gluten-exclusion and of coeliacs to the exclusion of one of the other dietary antigens have not been performed.

The lack of response to milk antigen in this study confirms previous work (Haeney & Asquith, 1978), though a relatively low concentration of milk was used. Higher concentrations did not produce toxicity but plates were difficult to read because of cloudiness.

In this case there may be an argument for repeating the studies using purified milk proteins such as lactoglobulin.

The lack of effect of HLA-B8 on immune response to these other dietary antigens is interesting. The increased response to gluten seen with HLA-B8 could be explained as merely a facet of the generalised increase in immune responsiveness known to be associated with possession of this antigen (Vladutiu & Rose, 1974) but the lack of an increased response to these other antigens makes this less likely. This suggests that the response to gluten is specifically increased in HLA-B8 individuals and may therefore be of some pathogenetic significance. Interestingly, these findings are again paralleled by earlier antibody studies (Scott et al. 1976) where HLA-B8 was associated with an increased titre of antibodies to gluten but not to other dietary antigens.

The LMI test in coeliac disease has been widely interpreted as giving a quantitative estimation of cell-mediated immunity to gluten or its subfractions. Positive results have then been used to implicate a type IV or delayed hypersensitivity reaction to gluten in the pathogenesis of the mucosal lesion. However, the original justification for the use of the LMI test

as an assay of cell-mediated immunity lies in the correlation between results of the test and results of skin tests for delayed hypersensitivity. Skin testing with gluten fractions in coeliac disease has not supported the presence of delayed hypersensitivity reactions, in contrast to the good correlation seen between skin tests and LMI tests when antigens such as PPD are studied for which the importance of cell-mediated immunity is clear (Rauch & King, 1973; Mitchell et al. 1972; Rosenberg & David, 1970). Even in these systems, however, there is considerable evidence to show that the concept of an antigen reacting with a specifically sensitised T lymphocyte which then releases a lymphokine which in turn inhibits polymorph migration is an over-simplification and that other mechanisms may play a part. Rocklin (1974) showed that direct migration inhibition would occur in a population of mononuclear cells with 10-15% added polymorphs only if the polymorphs were derived from a donor sensitised to the antigen used. This suggested that some factor on the surface of the polymorphs (possibly a cytophilic antibody) was participating in the inhibition. Senyk & Hadley (1973) found that purified mononuclear cells were unaffected in the LMI test but that purified polymorphs were inhibited by

antigen, again suggesting a role for cytophilic antibody. Polymorphs may also be rendered susceptible to non-specific (and presumably non-immunological) inhibition. Clausen (1974) showed a transient sensitivity to Brucella antigens without delayed hypersensitivity in patients with streptococcal tonsillitis. Inhibition of migration by factors other than T-cell produced lymphokines has already been discussed in the Introduction.

Several groups have demonstrated blocking of migration inhibition by protein synthesis inhibitors using antigens (BCG and PPD) accepted as lymphokine producers (Rosenberg & David 1970; Gorski, 1974; Maini et al. 1973). These results suggest that protein synthesis is necessary for lymphokine-mediated migration inhibition. The exact mechanism of action is not understood, though there is some evidence to suggest that the puromycin is acting on the target cell to render it unresponsive to lymphokine rather than on lymphokine release by the lymphocyte (Kotkes & Pick, 1975). The failure of puromycin to block inhibition in this study thus argues against the role of lymphokines in migration inhibition produced by gluten. The concentration of puromycin used is similar to that used in previous studies and is shown to be sufficient by

its effect on migration index to PPD in the Mantoux-positive controls. The lack of effect with gluten is in accord with earlier work showing that jejunal biopsies from coeliac patients continued to produce soluble inhibitors of leucocyte migration when cultured with gluten despite the presence of puromycin (Howdle et al. 1981).

The results of the LMI test performed with purified sub-populations of leucocytes support the view that a non-lymphokine mechanism is involved. Technically, these experiments are far more demanding than the simple LMI test and the number of tests done was much smaller. Nevertheless, the cell separation techniques worked satisfactorily and results seem clear. The demonstration that purified polymorphs from coeliac patients are inhibited by gluten is strong evidence against the role of lymphokine in producing migration inhibition. No such inhibition was seen using PPD in the Mantoux-positive subjects. The addition of T-lymphocyte enriched cell suspensions failed to increase the degree of migration inhibition, again suggesting that lymphocyte products are not involved. This again contrasts with the findings with PPD where addition of the T-cell preparation produces migration inhibition in the Mantoux-positive subjects.

Furthermore, the correlation between results of the LMI test performed in the standard way and that performed with polymorphs alone suggests that, though lymphocyte activation may occur with gluten in the context of this test, it is not the major mechanism by which migration inhibition occurs.

The sensitisation of normal leucocytes to gluten-induced migration inhibition by pre-incubation with serum from coeliac patients suggests that cytophilic antibody may be involved. Inhibition of leucocyte or macrophage migration by cytophilic antibody in the presence of antigen has been shown by several groups of workers (Heise et al., 1968; Kostiala & Kosunen, 1972; Lockshin et al., 1973; Amos et al., 1967; Wasserman, 1965; Ortiz-Ortiz et al., 1974). The existence of antibodies which bind to phagocytic cells and modify their immune capabilities has been known for many years (see review by Tizard, 1971). Most of the available experimental data concern macrophage-cytophilic antibody (Tizard, 1971; Berken & Benacerraf, 1966; Nelson & Boyden, 1967) though similar antibodies which bind to polymorphs and modify their antigen-binding and phagocytic capabilities have been described in animals (Fidalgo & Najjar, 1967; Watson, 1976) and there is

some evidence that the IgG subclasses IgG₁ and IgG₃ in man bind to polymorphs as well as to monocytes (Hay et al., 1972).

Cytophilic antibody binds to cells via interaction between its Fc portion and Fc receptors on the cell surface (Berken & Benacerraf, 1966). The blocking of the sensitisation of normal leucocytes by coeliac serum by incubation in the presence of aggregated IgG (which blocks Fc receptors) prior to exposure of the cells to the coeliac serum supports the contention that cytophilic antibody is involved. Immune complexes, also reported to cause migration inhibition (Spitler et al., 1969; Kotkes & Pick, 1975), are unlikely to be the cause of the inhibition of the normal leucocytes as their migration in control medium was unaffected by incubation with coeliac serum, inhibition only being seen when gluten was added.

The other experiment undertaken to investigate possible other mechanisms of migration inhibition was that involving rhamnose. This showed no effect and thus did not support the hypothesis that migration inhibition may be mediated via a lectin-like binding of gluten to the leucocyte cell surface. This does not, of course, invalidate this hypothesis, but to do so would require screening of a wide range of possible

inhibitory sugars. As the only evidence available (Douglas, 1976) suggests that rhamnose is the likely sugar specificity of the putative lectin this seems a reasonable starting point. Douglas' results, however, are unconfirmed and show certain unexplained features (such as the increased binding shown with some other sugars) and it is not clear that further investigation is warranted in the absence of further supportive background evidence.

How do these new observations on the mechanisms of migration inhibition in the direct LMI test relate to the conclusions shown from earlier published work and the results presented earlier in this thesis? First, it is essential to emphasise that these results must only apply to the LMI test performed as described here. There is no doubt that minor technical variations can influence the results of the LMI test considerably (Maini et al., 1973). Whether changes in factors such as the method of isolation of the cells (with possible consequent variations in the proportions of various sub-populations of white cells in the final cell preparation) might affect the mechanisms of migration inhibition is not known. However, it does seem possible that this may be so. There is certainly evidence that varying the crude proportions of

lymphocytes to polymorphs may affect the results (Warrington et al., 1976). However, results in the present studies are similar to earlier work both as regards the degree and prevalence of positive reactions with gluten in coeliac patients and are consistent with respect to the effects of dietary manipulation (Bullen & Losowsky, 1978b; Haeney & Asquith, 1978). These earlier results have all been interpreted as evidence of cell-mediated immunity to gluten and this interpretation must now be questioned. It is interesting that the limited information available on changes in anti-gluten antibody titres with dietary changes seems to suggest parallel changes in titre to the changes seen in LMI test results (Rossipal, 1974; Scott, 1975) and that humoral immune responses to gluten but not to other dietary antigens seem to be affected by possession of HLA-B8 in the absence of coeliac disease, though anti-gluten titres are not affected by HLA-B8 in coeliac patients (Scott et al., 1974; Scott et al., 1976). This is merely circumstantial evidence and there is no reason to suppose that titres of cytophilic antibodies would exactly follow those of antibodies detected in more conventional ways.

There is, of course, still much evidence to demonstrate that cell-mediated immunity to gluten exists. The indirect LMI test results of O'Farrelly et al. (1982) are impressive (though interestingly fail to show any effect of gluten-free diet on results) and this test has not been demonstrated to be affected by factors such as immune complexes or cytophilic antibody. However, suitable control experiments do not seem to have been performed. Further studies of the LMI test in any form should include studies to define the mechanisms involved. As discussed in the Introduction, the large number of lymphocyte transformation studies give varying results and there are some discrepancies in the time course of the responses. Despite this, however, it does seem probable that cell-mediated immunity to gluten can be demonstrated. Its exact relevance to pathogenesis, especially in the absence of skin test delayed hypersensitivity, remains uncertain. As for the possible pathogenetic role of cytophilic antibody, it is worth recalling the work of Ezeoke et al. (1974) suggesting that ADCC may be involved in producing jejunal mucosal damage and this approach merits further study.

It is also noteworthy that very recent work (Verkasalo, 1982) has demonstrated binding of a gliadin subfraction to 25-65% of B lymphocytes (i.e. those bearing surface immunoglobulin) from treated coeliac patients. Binding was unaffected by HLA status (though one normal control with DR3 showed 34% binding) and it was felt that it was improbable that such a large proportion of cells would be synthesising anti-gluten antibody. It is tempting to speculate that this binding could be mediated via cytophilic antibody on the surface of K cells which would segregate with B cells on density-gradient centrifugation.

Interpretation of the effect of diet on LMI results in the light of the present studies merely requires the assumption that the relevant antibody is fixed in the gut when a gluten-containing diet is taken and is released into the blood in increased quantity after a gluten-free diet is started. Results of the indirect LMI test with cultured jejunal biopsies are more difficult to explain. They may represent antibody synthesis in the mucosa of untreated coeliacs. Culture with gluten seems unlikely to increase release of free antibody but it is possible that immune complexes are formed and elute into the medium to be measured in the indirect test. This is highly speculative but is at least open to experimental investigation.

So far as studies with the other dietary antigens are concerned, there is no information as to whether these too are producing migration inhibition via cytophilic antibody or by another mechanism.

Jejunal biopsy culture studies

The studies reported in this thesis represent a preliminary effort to utilise the potential of the jejunal biopsy culture technique to elucidate possible immunological or other mechanisms of gluten-induced mucosal damage. Previously only Falchuk, Gebhard & Strober (1974) in their experiments involving co-culture of biopsies from untreated and treated coeliac patients and Katz et al. (1976) and Bramble et al. (1981) using steroids, have used this approach.

Much of the difficulty involved in using the system is in deciding what parameter should be used to quantify the degree of mucosal damage. Biochemical methods have been widely used with conflicting results. Falchuk's group in the U.S.A. have had considerable success using this method (Falchuk et al., 1974; Katz & Falchuk, 1978; Katz et al., 1976; Katz & Falchuk, 1978; Falchuk et al., 1980) and claim they can diagnose coeliac disease reliably. In other hands, however, results are less impressive. Hauri et al. (1978) and Howdle et al. (1981b) showed similar

increases in alkaline phosphatase activity of cultured biopsies from coeliac patients but could not demonstrate any effect of gluten. Other workers (Mitchell et al., 1974; Stevens et al., 1978) could not demonstrate any change in alkaline phosphatase, though Mitchell et al. (1974) showed an increase in enzyme activity in the culture medium. The explanation for these discrepancies is not clear. Minor differences in the technique of the biopsy culture may be responsible, but a more likely explanation has been suggested by Howdle et al. (1982). These workers point out that Falchuk's group have studied mainly children and adolescents, whereas other workers have mainly studied adult coeliacs and suggest that in vitro gluten sensitivity may be a function of age. They also point out that the known morphological patchiness of the mucosal lesion in coeliac disease (Scott & Losowsky, 1976c) may well be accompanied by varying gluten sensitivity of different areas of the mucosa. Falchuk's group culture separate biopsies in culture whereas Howdle et al. compare pieces cut from the same biopsy and cultured in different conditions.

In view of these varied findings it is perhaps not surprising that alkaline phosphatase levels in the present study did not demonstrate any significant

effects of culture with gluten and lymphocytes. Mean pre- and post-culture values of alkaline phosphatase activity were similar to those reported by other workers and the rise in activity with culture is also shown. The lack of any effect of gluten alone is in accord with all previous work which shows the relative insensitivity of treated coeliac mucosa to in vitro damage. The various methods of expression of results do not affect these conclusions.

Of much greater interest are the results of the studies using quantitative histology to assess mucosal damage. These demonstrate that the combination of gluten and autologous peripheral blood mononuclear cells can produce significant mucosal damage to jejunal biopsies from treated coeliac patients in vitro. The damage produced is greater than that produced by culture with either gluten or lymphocytes alone, suggesting that there is some synergistic effect. The lack of effect of this combination on the cultured biopsies from non-coeliac patients suggests that the effect is specific to coeliac disease.

The demonstration of significant effects by use of quantitative histology in the absence of changes of alkaline phosphatase activity is in accord with recent published work, which suggests that histological

methods may be more sensitive than biochemical methods (Howdle et al., 1981a). In this study great care was taken in the technique of measuring enterocyte height and the method undoubtedly gives reproducible results. On the basis of the reproducibility studies one can calculate that changes in enterocyte height of more than around 1.3μ are unlikely to arise from observer error. This would represent a change of about 5% in enterocyte height. Both gluten alone and lymphocytes alone did produce small reductions in enterocyte height (of around 3%) which were not statistically significant. The 9.1% reduction produced by the combination of gluten and lymphocytes is significant compared with both biopsies cultured in control medium and those cultured with gluten or with lymphocytes alone. If, however, the changes produced by gluten alone and by lymphocytes alone are added, then that produced by the combination no longer differs significantly, though the mean change is almost double ($t = 0.542, p > 0.1$). The case for synergy rather than merely an additive effect of two separate non-specifically noxious influences thus remains not proven by these experiments. Nevertheless, the relative magnitude of the changes seen and the lack of an effect in the non-coeliac biopsies suggests that some synergistic effect is occurring.

The lack of a significant toxic effect of gluten alone conflicts with the results of Howdle et al. (1981a) who showed a 5% reduction in enterocyte height in biopsies from treated coeliac patients cultured with gluten fraction III. This difference does not seem to be explicable on the basis of patients at different stages of treatment, as the mean pre-culture enterocyte height of patients in the two studies is very similar. Two factors are probably important: (1) the smaller numbers in the present study and (2) the fact that Howdle et al. were comparing pieces from single biopsies which were cut into two and cultured with and without gluten. The increased number of culture conditions in the present study precluded such an approach, necessitating comparisons between biopsies from different sites and thus possibly reducing the precision of the method.

The increased sensitivity to gluten of the biopsies cultured with lymphocytes fits nicely with the hypothesis that the number of gluten-reactive lymphocytes in the jejunal mucosa declines when gluten is excluded from the diet and these cells are then free to circulate in the blood. Co-culture of biopsies and peripheral blood lymphocytes then allows the lymphocytes to react with the gluten peptides and

damage the mucosa. There are several possible mechanisms by which the lymphocytes may produce mucosal damage. These include generation of cytotoxic T cells, production of lymphokines and antibody-dependent cellular cytotoxicity (ADCC). Cytotoxic T cells are activated by cell-surface determinants (including histocompatibility antigens) (Zinkernagel & Doherty, 1979) and damage by such a mechanism would presuppose binding of the gluten peptide to the cell surface. Of the numerous lymphokine activities described, lymphotoxin is directly toxic to cultured cell lines. Lymphokine-mediated damage does not necessarily involve binding of gluten to the enterocyte, which may be damaged as an "innocent bystander" as described in graft-versus-host disease (Elson et al., 1977).

ADCC also involves binding of the peptide to the enterocyte surface. Specific antibody then binds to the peptide and a variety of cells with receptors for the Fc portion of the antibody molecule (including macrophages, polymorphs and the small lymphocyte-like K cell) may then be involved in producing the cytotoxic effect (Roitt et al., 1976). None of these mechanisms involve complement, which was not present in the culture system in these studies. The present results are consistent with any of these mechanisms and further

experiments involving culture with sub-populations of lymphocytes, with protein-synthesis inhibitors to block lymphokine action and with addition of aggregated IgG to block ADCC are needed to define the mechanism further. The studies of Falchuk et al. (1974) involving co-culture of biopsies of untreated and treated coeliacs are also relevant. These suggested that a soluble mediator was involved and would support lymphokine-mediated or ADCC mediated damage.

It is also interesting to speculate on how the results discussed earlier relating to the mechanisms of the LMI test may fit in. These would support the concept of coeliacs possessing antibody to gluten capable of binding to cells known to be involved in ADCC. In addition, the work of Ezeoke et al. (1974) and the nature of the early histological changes seen after gluten challenge (Anand et al., 1981) would suggest that greater attention be paid to ADCC as a possible pathogenetic mechanism in coeliac disease.

The lack of correlation between the degree of damage produced by culture with gluten and lymphocytes and the result of the LMI test could have several explanations. It may merely be that the two methods are so imprecise that any correlation is missed. Alternatively, if the LMI test is measuring cytophilic

antibody mainly on polymorph cell membranes then it is not surprising that results do not correlate with the putative cytotoxic effects of blood mononuclear cells, which may either have a different affinity for the cytophilic antibody or may be exerting their effect through another mechanism. If cytophilic antibody is involved, it may be being synthesised in the mucosa and the added lymphocytes may merely provide a source for recruitment of effector K cells. Clearly there is great scope for further experimentation in this field to define the mechanisms involved.

The negative results of the experiments involving assay of phosphatidyl inositol in cultured jejunal biopsies are perhaps not surprising, for several reasons. First, the assay itself is relatively complex and considerable difficulty was encountered in modifying it for use in an organ culture system, though the final system did seem to give fairly consistent results. Second, as no satisfactory system exists for culture of isolated enterocytes the assay had to be applied to cultured biopsies. As all cell membranes metabolise phospholipids, any small change confined to the enterocytes is liable to be swamped by the background turnover in all the other cells in the biopsy. Finally, the original postulate, i.e. that a

lectin-like binding of gluten to the enterocyte membrane might affect turnover of this lipid, may be incorrect. Nevertheless, the experiments do demonstrate that the jejunal biopsy culture system can be used to study aspects of cell-membrane metabolism and it is possible that this approach may yield significant results in coeliac disease. This would be made much more likely if a reliable culture system for isolated enterocytes were developed.

CONCLUSIONS

Several important conclusions can be drawn from the work presented in this thesis.

- 1) Results of the direct LMI test are influenced by the histocompatibility antigen HLA-B8 in a way which suggests that an increased immune response to gluten (or even an immune response gene for gluten) may be necessary but is not sufficient for development of the disease. The test should not be used diagnostically without allowance for the effect of HLA-B8.
- 2) Results are affected by diet in a manner compatible with the theory of sequestration of gluten-reactive cells or antigluten antibody in the gut by gluten in the diet, release of these by gluten exclusion and later decline in immunity after prolonged antigen exclusion.
- 3) Positive results with the LMI test in coeliac disease are not only found with gluten but also with other dietary antigens - a position similar to that found with serum antibodies. This weakens the argument for the pathogenetic importance of such responses.
- 4) The direct LMI test is not an index of T cell mediated immunity but seems primarily to measure cytophilic antibody.

- 5) Co-culture of jejunal biopsies from treated coeliacs with autologous lymphocytes restores gluten sensitivity in vitro - a finding compatible with the theory outlined in (2) and supporting the role of immunity to gluten in pathogenesis.
- 6) No support is found for the lectin theory of toxicity.

It is customary to end the discussion of a body of research results with an attempt to combine any new results with available information in an all-encompassing theory. However, though the conclusions above can be drawn from the results presented in this thesis, I do not feel that further theorizing is particularly helpful. If abnormal immunity to gluten is a central pathogenetic mechanism in coeliac disease, and this is not yet proven, then it is likely that the abnormality will involve more than one of the possible immune mechanisms discussed in the Introduction and, most likely, the relative importance of the different mechanisms will be different at different stages in the natural history of the disease. The results presented here draw attention to one potential immunopathogenic mechanism which has perhaps been rather neglected by investigators, and also demonstrate that the organ culture system may be useful in investigating the mechanisms involved.

Of possibly greater importance, however, is the demonstration that immunological tests cannot be assumed to have the same basis in one disease as they have been shown to have in another, and that it is necessary to go back to basic immunological methods to avoid misinterpretation of results which, in the long run, may hamper rather than help our understanding of the precise pathogenic mechanisms involved in this disease.

DECLARATION OF ORIGINALITY

I hereby declare that the work reported in this thesis was originated and performed by me, with the exception of technical assistance detailed in the Acknowledgments. Furthermore, I declare that this thesis has been entirely composed by me.

A handwritten signature in black ink, appearing to be 'K. Wang', is written above a solid horizontal line.

Publications arising from work contained in this thesis

HLA-B8 and cell-mediated immunity to gluten.

F. G. Simpson, A. W. Bullen, D. A. F. Robertson
& M. S. Losowsky.

Gut, 1981, 22, 633-636.

Cell-mediated immunity to dietary antigens in
coeliac disease.

F. G. Simpson, D. A. F. Robertson, P. D. Howdle
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Jejunal biopsy and lymphocyte co-culture in
coeliac disease.

F. G. Simpson, P. D. Howdle, D. A. F. Robertson
& M. S. Losowsky.

Scandinavian Journal of Gastroenterology (in press)

The Leucocyte Migration Inhibition Test in Coeliac
Disease - A reappraisal.

F. G. Simpson, H. P. Field, P. D. Howdle,
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Appendix Table 1

Duplicate results of original assay for PI turnover
(cpm/mg)

<u>Duplicate 1</u>	<u>Duplicate 2</u>
106.7	150.4
126.8	134.2
66.9	101.1
66.7	74.5
80.5	82.9
128.5	102.3
164.5	246.5
52.9	110.9
79.3	101.2
102.3	103.2
27.9	187.0
88.5	162.0
102.7	67.6
53.2	89.5
59.2	72.4
94.5	91.9
69.3	77.6
94.0	315.8
131.1	276.5
448.0	751.2
840.8	578.0
706.0	1998.5
813.9	608.0

$$SD = \sqrt{\frac{\sum d^2}{n}} = 208.1$$

$$\text{Mean} = 238.9$$

$$CV = 87.1\%$$

Appendix Table 2

Duplicate results for modified assay for PI turnover
(dpm/mg protein)

Duplicate 1

Duplicate 2

81.4	89.9
91.8	85.5
87.5	85.0
96.4	92.6
88.5	98.9
61.3	67.0
89.0	87.0
126.8	119.4
90.1	87.0
65.9	72.4
59.9	57.5
43.6	42.8
88.6	64.1
59.5	49.1
80.9	100.6
71.8	73.7
41.8	44.1
39.2	46.6
63.4	70.5
41.0	39.2
41.0	42.3
93.2	82.6
64.5	63.5
51.7	49.7
74.9	78.2
78.0	70.0
85.9	85.5
43.5	39.1
77.3	78.0
73.0	86.0
89.5	99.3
93.5	75.2
111.3	113.9
159.3	157.9
129.8	120.5

$$SD = \sqrt{\frac{\sum d^2}{n}} = 5.99$$

$$\text{Mean} = 77.85$$

$$CV = 7.7\%$$

Appendix Table 3

Quench curve for PI turnover assay

$$N = N_0 \exp\left(\frac{0.693t}{t}\right)$$

$$\therefore 209,500 = N_0 \cdot 1.1187$$

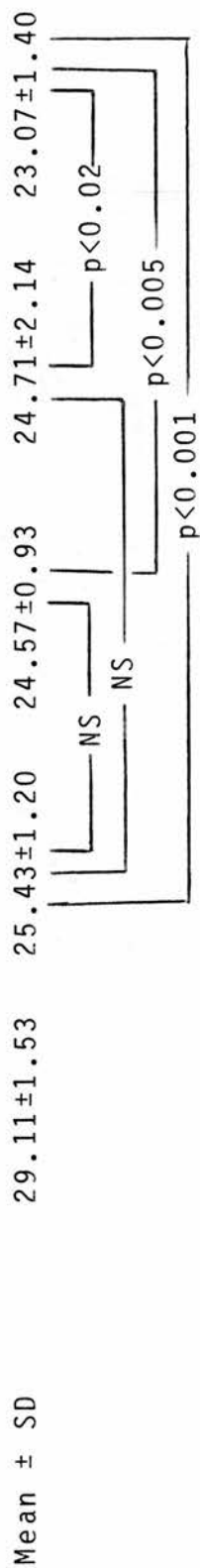
$$N_0 = 187,624$$

	<u>External Standard Ratio</u>	<u>Counts per minute(SD)</u>	<u>Efficiency(%)</u>
Standard	.6568	67739.8(0.2)	36.1
+ 1 μ l CCl ₄	.6391	65079.0(0.2)	34.7
+ 2 "	.6069	61545.0(0.2)	32.8
+ 3 "	.5962	58349.5(0.2)	31.1
+ 4 "	.5675	54351.5(0.2)	29.0
+ 5 "	.5361	50658.5(0.2)	27.0
+ 6 "	.5160	47266.5(0.3)	25.2
+ 7 "	.4846	44339.5(0.3)	23.6
+ 8 "	.4479	41584.5(0.3)	22.2
+ 9 "	.4295	38800.0(0.5)	20.7
+10 "	.4106	36930.0(0.5)	19.7
+11 "	.3669	33306.0(0.5)	17.8
+12 "	.3482	31002.5(0.5)	16.5

Appendix Table 4

Enterocyte heights (μm) of biopsies from coeliac patients pre-culture and after 24 hour culture in control medium, with added lymphocytes, with added gluten fraction III (GFIII) and with added lymphocytes and added GFIII.

<u>Patient</u>	<u>Pre-Culture</u>	<u>Control</u>	<u>+Lymphocytes</u>	<u>+ GFIII</u>	<u>+Lymphocytes +GFIII</u>
1	27.20	26.05	22.75	24.32	23.50
2	26.95	23.50	23.90	23.55	21.80
3	29.20	25.10	25.85	24.60	24.40
4	31.05	23.85	25.25	27.00	25.20
5	28.50	24.55	23.90	23.75	24.05
6	29.20	26.75	24.70	24.50	23.19
7	26.95	24.90	23.85	28.15	23.05
8	29.00	26.90	24.25	28.35	21.75
9	26.25	24.75	25.90	21.55	21.50
10	29.95	25.95	25.25	25.80	23.20
11	31.00	26.60	24.15	23.00	23.00
12	30.80	27.10	25.50	24.75	24.85
13	28.70	24.65	24.10	21.85	20.41



All post-culture groups have significantly lower enterocyte heights than the pre-culture values ($p < 0.001$)

Appendix Table 5

AP/mg prot.

PATIENT	PRE	C	CL	G	GL
1	45.9	88.2	151.3	90.2	85.4
2	55.2	111.8	82.9	149.4	81.2
3	31.6	33.7	56.4	29.5	44.6
4	26.6	84.3	63.7	101.7	105.4
5	15.5	26.3	24.3	50.7	45.2
6	18.4	87.1	70.3	26.7	32.5
7	65.6	56.6	17.3	38.6	14.1
8	32.8	49.1	79.4	50.5	38.5
9	20.7	64.8	93.9	43.0	64.7
10	79.4	58.1	78.1	76.1	67.2
11	57.6	40.9	59.8	34.0	65.3
12	35.3	63.9	46.2	75.7	66.5
13	13.6	13.2	11.0	10.6	4.4
MEAN	38.3	59.9	64.2	59.8	55.0
SD	20.8	27.9	36.9	37.9	28.6

Appendix Table 6

AP/mg. wet wt.

PATIENT	PRE	C	CL	G	GL
1	1.46	2.54	4.34	2.15	2.51
2	1.37	2.51	3.19	2.32	1.21
3	1.24	1.38	2.58	2.12	3.33
4	1.32	2.91	3.53	4.68	4.48
5	0.37	0.37	1.01	1.18	0.79
6	1.33	3.97	3.25	1.29	1.19
7	1.25	2.59	1.33	2.15	1.17
8	1.64	2.90	4.92	3.30	1.92
9	0.80	1.80	1.88	0.88	2.61
10	2.63	2.88	2.88	3.29	3.18
11	2.30	1.46	1.79	0.63	1.37
12	2.00	3.34	2.10	4.37	5.76
13	0.52	0.56	0.80	0.77	0.36
<u>MEAN</u>	<u>1.40</u>	<u>2.25</u>	<u>2.58</u>	<u>2.24</u>	<u>2.30</u>
SD	0.65	1.07	1.26	1.33	1.57

Appendix Table 7

AP/ μ g DNA

PATIENT	PRE	C	CL	G	GL
1	1.43	1.64	3.88	1.93	0.65
2	1.65	2.83	2.00	3.52	1.23
3	0.85	0.54	1.46	0.77	0.60
4	1.83	1.93	3.13	3.51	3.58
5	0.85	0.71	0.90	2.28	1.34
6	2.32	3.78	3.06	3.36	3.56
7	1.07	1.20	0.71	1.05	0.30
8	0.89	1.26	1.74	1.21	0.88
9	0.66	1.03	1.78	0.94	1.16
10	1.10	1.36	1.86	1.40	1.40
11	0.96	0.57	1.37	0.35	0.83
12	1.46	2.20	1.63	3.35	4.09
13	0.40	0.62	0.60	0.41	0.08
MEAN	1.19	1.51	1.86	1.85	1.52
SD	0.53	0.97	0.98	1.22	1.33