IMMUNOLOGICAL STUDIES IN DERMATITIS HERPETIFORMIS

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ABSTRACT

Using indirect immunofluorescence antibodies against components of connective tissue were found in patients with dermatitis herpetiformis (DH) and with childhood and adult coeliac disease. Two main patterns of immunofluorescent staining were seen, and the antibodies appeared to be directed against antigens of 'reticulin' fibres as detected histochemically by silver staining. These antibodies were therefore called 'anti-reticulin antibodies' (ARA). They were found in highest incidence in children with coeliac disease where they were of greatest use diagnostically. They were also found more frequently in patients taking a gluten-containing diet and disappeared from the circulation on gluten-withdrawal. Absorption studies using gluten and gluten-fraction 3 suggest that there may be cross-reactivity between gluten and reticulin.

Using direct immunofluorescence IgA deposits in the clinically uninvolved skin were found in all 50 DH patients examined, but in none of the adult coeliac disease subjects. It is suggested that the presence of these IgA deposits should be used as a major criterion in the diagnosis of DH. Two main patterns of IgA deposition -'papillary' and less commonly, 'continuous' - were seen. From comparative histological studies, these IgA deposits appear to be located in the reticulin fibres in the dermal papillae. Complement deposits, as C3 but not C1q were found in the same situations as the IgA deposits. It is suggested that the skin lesions in DH are the results of complement activation - and that IgA is the agent responsible in

Abstract - contd.-

activating complement via the alternate pathway of complement fixation. The possible cross - reactivity of reticulin and gluten may be of relevance in this situation.

Marked lymphocytic infiltration of the small-intestinal epithelium was found in almost all the DH patients. This lymphocytic infiltration was most marked around the epithelial cell nucleus and fell with gluten-withdrawal. It is suggested that their presence is due to an immunological response to gluten and that this infiltration is evidence of gluten-sensitive enteropathy at a microscopic level. The finding of raised intraepithelial lymphocyte counts in the majority of the DH patients suggests that virtually all these patients have gluten-sensitive enteropathy.

These results would support the concept that DH is a single disease entity with dermatological and gastrointestinal manifestations. Such a concept is also supported by the HL-A studies reported here which show that patients with DH have a high incidence of HL-A 8 as found in adult coeliac disease, and that the incidence is similar in those patients with or without macroscopic mucosal abnormalities. The skin rash of DH may respond to a gluten-free diet and it is suggested that the dermatological manifestations of this disease should be called 'gluten-sensitivity of the skin'.

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DEDICATION

This thesis is dedicated to

MARY YAT HUAN

and to

MY PARENTS

PART 1:

THE PROBLEM

THE PROBLEM

Dermatitis herpetiformis (D.H.) is a chronic blistering skin condition of unknown aetiology. For many years, treatment has been the empirical administration of sulphones (dapsone) or sulphonamides, both of which have a dramatic effect on the skin rash. The discovery of a small-intestinal abnormality in patients with D.H. in 1966, and the subsequent finding that it is due to gluten sensitive enteropathy as found in coeliac disease, has opened up new perspectives to this disease and this may eventually prove to be a major factor in the attempt to elucidate the aetiology and pathogenesis of D.H.

There is now increasing evidence of immunological abnormalities in both D.H. and coeliac disease. This thesis aims to look into the immunological aspects of D.H. In many instances, parallel studies have been done in patients with coeliac disease. From these studies, an attempt will be made:-

(i) to assess the relevance of these findings in the aetiology of D.H.

(ii) to explain the pathogenesis of the skin lesions in D.H.

(iii) to assess the role of gluten in the aetiology of both D.H. and coeliac disease.

(iv) to determine why patients with D.H. have skin lesions whilst patients with coeliac disease do not.

(v) to assess the diagnostic implications of these findings.

(vi) to assess the therapeutic implications of these findings.

PART II

INTRODUCTION

INTRODUCTION

Historical Aspects.

The term dermatitis herpetiformis (D.H.) was first used by Duhring in 1884 to describe a group of polymorphous bullous skin conditions which had previously been called by different names and which were distinct from pemphigus, eczema and herpes. Hence, in his book "Cutaneous Medicine - A Systemic Treatise on Diseases of the Skin" (1898), Duhring pointed out that the eczema puriginosum and herpes circnatus bullosus of Erasmus Wilson, the hydroa vacciniform of Bazin and the hydroa herpetiforme of Tilbury Fox were variations of what was essentially a single disease process. This affection appears to have been first recognised and was clearly described by Tilbury Fox in 1880 ("A Clinical Study of Hydroa") though his claims to priority in this matter have largely been overlooked in both America and Europe.

In his original report in 1884, Duhring considered D.H. to be a protean disorder and named six varieties of the disease for which he proposed the name "Dermatitis Herpetiformis" - namely erythematosa, vesiculosa, bullosa, pustulosa, papulosa and multiformis. It is perhaps best to express in his own words his arguments for the concept that these are variations of the same disease process:

- "In all instances the disposition to group or to extend about the periphery was more or less marked."
- "Itching, burning or pricking sensations are always present."
- "The tendency is in almost every instance that I have observed, to multiformity."
- "There is moreover, in almost every case, a distinct disposition for one variety, sooner or later, to pass into some other variety."
- "... nor in any case, has the disease proved fatal."

The views of Duhring were greatly supported by the French author Brocq in his paper "Note sur les dermatites polymorphes douloureuses" in 1898. Brocq emphasized that the skin lesions in D.H. were frequently polymorphic and not simply 'blisters'. The views of these two authors were soon generally accepted and about the turn of the century, the disorder became known under the name "Duhring-Brocq's" disease. Whilst Duhring commented mainly on the 'herpetiform' nature of the disease, Brocq stressed the 'polymorphic' nature of it.

Following on these original descriptions the term Duhring-Brocq was used extensively in describing this blistering condition. However, the existence of other blistering conditions - in particular pemphigus, bullous pemphigoid and erythema multiforme - led to much confusion as to whether these were all variants of a single disease process or separate distinct entities. The separation of pemphigus into a distinct entity based on the finding of an intraepidermal blister and acantholytic cells (Civatte 1943) somewhat simplified the situation. Lever (1953) coined the term pemphigoid to distinguish patients who on clinical grounds were thought to have pemphigus, but on microscopy were found to have subepidermal blisters. The definition of the blistering disorders showing histologically subepidermal blisters - which include D.H. and bullous pemphigoid - therefore remained largely unresolved. Pierard et al. (1957) defined certain histological criteria for the diagnosis of D.H., and considered the presence of papillary micro-abscesses in the early skin lesion to be distinctive for the disease and diagnostic for D.H. These findings were confirmed by Pierard and Whimster (1961) and MacVicar et al. (1963). Subsequent studies, however, indicate that these are not exclusive to D.H. and that such micro-abscesses can be found in other conditions including bullous pemphigoid (Jablonska and Chorzelski 1962, Lever 1965, Connor et al. 1972). The separation of

D.H. and bullous pemphigoid into separate entities has had to await the advent, in the last decade or so, of immunofluorescent methods in the study of skin diseases, which show definite differences. However, it is still not generally accepted that the two conditions are entirely distinct but that there is sometimes an overlap. It would be safe however to say, that most people would now accept that the original descriptions of Duhring and Brocq comprise two entities - namely D.H. and bullous pemphigoid.

Clinical Aspects:

Morphology

D.H. is characterized by an intensely irritating polymorphous eruption affecting mainly the pressure points. Hence, the elbows, neck, shoulders, axillary folds, scapulae, buttocks and knees are commonly affected sites, but no area is spared and the lesions may be found anywhere, including the mouth. As originally described by Duhring and substantiated by Brocq, the rash is frequently polymorphic. The intense attendant irritation often causes the patient to scratch and the so-called typical picture of blisters situated on an urticarial base are seldom seen. Instead, grouped excoriated papules or urticarial lesions are more common and the distinction from papular urticaria has to be made. Occasionally, the patient may present with lichenified red, scaly plaques - the result of continual scratching - and differentiation from eczema is difficult. Very widespread blisters are rare and may be confused with pemphigoid or erythema multiforme.

Incidence

Reports of the incidence of D.H. are few and vary between 1 : 200 (Bjornberg and Hellgren 1962), 1 : 500 (Eyster and Kierland 1951) and 1 : 800 (Everall 1954).

Age of Onset.

D.H. is essentially a disorder of adults. The most common age of presentation is in the third and fourth decades (Tolman et al. 1959, Bjornberg and Hellgren 1962, Smith 1966, Van der Meer 1972), but the disorder

may also appear for the first time in later adult life. However, it is also apparent that a small number of patients do develop skin lesions in the first and second decades which are similar to those seen in adults. In the past these have been termed juvenile D.H. or juvenile pemphigoid, and the question arises as to whether this is the same disorder as in adults or a different entity. The characteristic clinical features of the juvenile type of D.H. described by Sneddon (1972) are certainly different to those of the disorder as seen in adult life; the course of the disorder is different in that it tends to clear after 3 - 4 years (Kim and Winkelmann 1961), and the response to sulphones is variable, unlike the adult type (Sneddon 1972). It is probable that patients with so-called juvenile D.H. do in fact represent a group of disorders as suggested by Jablonska et al. (1971), and not one nosological entity. These workers based their results on immunofluorescent studies of the skin on 8 patients, 3 of whom showed IgG in the basement membrane (as found in pemphigoid), 2 showed IgA in the dermal papillae (as found in D.H.), and 3 showed no Ig deposits in the skin.

Likewise, a study on the small intestine of patients with juvenile D.H. in Jamaica (Warner et al. 1972) showed evidence of enteropathy on the macroscopic appearance of the mucosa in 5 of 10 patients, but not in all patients as suggested by Fry et al. (1972). However, Warner et al. (1972) did not carry out quantitative lymphocyte counts on the small intestinal epithelium, which Fry et al. (1972) found to be the most reliable indicator of an enteropathy. Thus, the true incidence of enteropathy in this series is not known.

In conclusion, it appears that present evidence based on clinical features, response to sulphones, immunological and gastrointestinal findings, suggest that a small proportion of patients with juvenile D.H. do in fact have the same disorder as D.H. seen in adults.

Sex Ratio

D.H. shows no prediliction for either males or females. There are reports of preponderance in males (Evans and Fraser 1961, Smith 1966), whilst others have found an equal distribution (Bolgert and Chastenet 1963), and yet others a preponderance in females (Degos and Touraine 1961).

Natural History

The majority of authors who have looked into this aspect of D.H. consider it to be a chronic disorder. In fact, Duhring (1884) in his original paper described the disorder as 'extending over years, persuing an obstinate, emphatically chronic course'. Everall (1954) however, stated that a third of patients are likely to be free of lesions after 10 years. Shuster and Marks (1970) stated that the duration of the disorder was only 4 years, but no other authors agree with this figure. In a retrospective study, Smith (1966) found spontaneous remission in only 7 of 149 patients, and the mean duration of the illness to be 44 months. It is likely that Duhring was right in his original description of the disorder and that it is chronic.

Treatment

Until recently, the treatment for D.H. has always been empirical, as the pathogenesis for this disorder was unknown. The first really helpful drug in controlling the eruption was sulphapyridine (Costello 1940). The drug was given at that time as it was thought that the disorder was an 'allergic' reaction to bacterial infection. Subsequently, sulphones which had been synthesized for the treatment of mycobacterial infections were shown to be effective in controlling the eruption of D.H. (Esteves and Brandao 1950, Cornbleet 1951). The mechanism of action of sulphones in D.H. is not understood, but the remarkably quick response of the rash and its recurrence on withdrawal of the drug, has led to the use of this therapeutic effect in the last twenty years as a diagnostic criterion for D.H. However, it is now known that a number of other skin disorders also improve with sulphones. These include vasculitis (Wells 1969, Thompson et al. 1971), erythema elevatum diutinum (Vollum 1968, Cream et al. 1971),

pemphigus (Floden and Gentele 1955, Winkelmann and Roth 1960, Seah et al. 1973), papular urticaria (Hewitt et al. 1971) and subcorneal pusutlar dermatosis (Sneddon and Wilkinson 1956). Thus the clearance of a rash with dapsone is not diagnostic of D.H. With the conclusive evidence that patients with D.H. have a gluten sensitive enteropathy (Fry et al. 1968, 1969, 1972, Shuster et al. 1968), a gluten-free diet has now been shown to clear the eruption (Fry et al. 1969, 1973).

Histological Aspects:

Light Microscopy

Following the observations of Civatte (1943) that pemphigus was a disease characterized by an intraepidermal blister and acantholysis, it has been possible to define some of the histological features of other bullous disorders. Civatte (1943) stated that his findings supported (as indeed they did) the original concept of Duhring (1884), that D.H. was a completely different disorder. Civatte's studies thus differentiated pemphigus from D.H., pemphigoid and erythema multiforme which histologically have subepidermal blisters. An attempt to distinguish these 3 disorders at a histological level was made by Pierard et al. (1957) who stressed the finding of the intrapapillary microabscess at the tips of the dermal papillae in D.H. These findings were confirmed by Pierard and Whimster (1961) and MacVicar et al. (1963), and both these groups of workers stressed that one was more likely to find the papillary microabscesses if the biopsy was taken early in the development of the skin lesion, and preferably from an urticarial peribullous area rather than an area of skin containing the blister. However, the papillary tip microabscess is not exclusive to D.H. and has been found in pemphigoid (Jablonska and Chorzelski 1962, Lever 1965, Connor et al. 1972). The infiltrate in the papillary tip abscess consists essentially of polymorphs and eosinophils. Later in the disease process it would appear that the polymorphs are destroyed, and that all that is seen is pyknotic nuclei and cellular debris, so-called nuclear dust (Lever 1965).

Eventually these papillary microabscesses appear to coalesce to form a small blister, and there is destruction of the basal lamina. Thus, D.H. is characterized by subepidermal bullae, papillary microabscesses and infiltration with eosinophils. These features however, are not pathognomonic of D.H.

A diagramatic representation of the differential diagnosis of the bullous disorders and the sites of pathology in the skin is shown in Figure I.1.

THE SMALL INTESTINE AND DERMATITIS HERPETIFORMIS

Until 1966 it was thought that D.H. was not particularly associated with any other disorder, though Smith (1966) in a review of the literature lists a number of conditions described in association with D.H. Many of the earlier descriptions of D.H. however, mention general ill health, malaise, loss of weight and mental symptoms, though no attempt was made to explain these features satisfactorily. Notably, Smith (1966) mentioned 2 patients with malabsorption in his retrospective study of 149 patients with D.H., and Bohnstedt and Ehlers (1965) described an increased incidence of disturbances of the digestive tract - usually an abnormality of gastric acidity.

Interest in the small intestine in D.H. was first aroused when in 1966 Marks et al. reported a high incidence of structural abnormality of the small intestine of patients with D.H. They found that 9 out of 12 patients had some abnormality of the small intestinal mucosa, but they were unable to come to any conclusion as to the possible cause of this finding. The following year, Fraser et al. (1967), Fry et al. (1967) and Van Tongren et al. (1967) reported a similar incidence of structural abnormality of the small intestine in patients with D.H. Neither Fraser nor Van Tongren and their respective colleagues came to any conclusion as to the cause of

the enteropathy. However, Fry et al. (1967) suggested that the intestinal abnormality may be due to gluten sensitivity as found in coeliac disease. Thus, in addition to the enteropathy, there was a high incidence of increased faecal fat excretion, of folate, iron and serum IgM deficiency, and of splenic atrophy. Further conclusive evidence was provided by Fry et al. (1968) who showed improvement in the abnormal faecal fat excretion, folate status, macroscopic appearance of the intestine, and surface epithelial cell height of the intestinal epithelium on treatment with a gluten-free diet (G.F.D.). On re-introduction of gluten into the diet, these changes were reversed, demonstrating the specificity of the pathogenic role of gluten in the enteropathy.

The incidence of the enteropathy in patients with D.H. was found in the initial series of reports (Fraser et al. 1967, Fry et al. 1967, Shuster et al. 1968) to be approximately 75%. The question was therefore quite rightly asked as to why 25% of patients with D.H. did not have an enteropathy, and were there in fact, two groups of patients with D.H., one with a gluten-sensitive enteropathy (G.S.E.), and another group without enteropathy. The question was answered 5 years later when it became apparent that the 75% incidence was due to inadequate criteria for diagnosing G.S.E. (Fry et al. 1972), and the fact that distribution of the enteropathy was patchy (Brow et al. 1971). It has been known for many years that one of the histological features of G.S.E. was infiltration of the submucosa and epithelium of the small intestine with lymphocytes. Ferguson and Murray (1971) found high intraepithelial counts of lymphocytes in the small intestine in coeliac disease but not in other gastro-intestinal disorders. Fry et al. (1972) then showed that there was quantitively increased lymphocytic infiltration in the small intestinal epithelium in over 95% of patients with D.H. irrespective of the macroscopic morphology of the mucosa. It was thus apparent that increased lymphocytic infiltration of the intestinal epithelium was a far more sensitive indicator and early

sign of G.S.E. than an abnormal macroscopic mucosal appearance which, until that time had been accepted as the most important criterion for diagnosing G.S.E. (Booth et al. 1962). Brow et al. (1971) using a multiple biopsy technique, where they took 6 to 8 biopsies from the small intestine, showed that 21 out of 22 patients with D.H. had evidence of enteropathy macroscopically, and that it was often patchy. They considered that this patchy distribution may explain the lower incidence of enteropathy in D.H. reported in previous series which were based on a single small intestinal biopsy. Taken together with the studies on lymphocytic infiltration of the epithelium (Fry et al. 1972), the incidence of enteropathy demonstrated is now over 95%. It would therefore seem probable that all patients with D.H. do in fact have enteropathy, and the few patients in whom no evidence has been found may well not have D.H., or it may be that the criteria for diagnosing G.S.E. are still inadequate. The concept that all D.H. patients have G.S.E. is further substantiated by the findings of Weinstein,(1973, 1974) who found that the presence of enteropathy is dose related to the amount of gluten ingested. Thus, he was able to induce morphological changes in the previously normal small intestine of D.H. patients by substantially increasing the amount of gluten in the diet over a period of a few weeks. Similar changes were not found in a normal subject taking the same diet.

Effect of Gluten-Free Diet on the Skin Lesions:

Following on the conclusive demonstration of a G.S.E. in D.H. (Fry et al. 1968, 1969, Shuster et al. 1968, Marks and Whittle 1969, Weinstein et al. 1971), considerable interest was focused on the skin rash in D.H. In particular, the question was raised as to whether gluten could be implicated in the pathogenesis of the rash, and whether treatment with a gluten-free diet (G.F.D.) clears the skin lesions.

Following trials of varying lengths of G.F.D., there have been conflicting

reports as to whether the skin lesions of D.H. improve and clear with a G.F.D. Fry and his colleagues (Fry et al. 1968, 1969) claimed early on that the skin lesions did respond to gluten withdrawal and there was clearance of the rash in a number of patients and recurrence of the lesions on the re-introduction of gluten. However, these claims were disputed by Shuster et al. (1968) and Weinstein et al. (1971). Subsequently convincing evidence relating gluten intake to the skin lesions was presented by Fry et al. (1973). These workers showed the presence of membrane-bound vacuoles at an ultrastructural level in the uninvolved skin in D.H. These were present in the skin in patients whose rash was controlled by dapsone, but not in the skin of patients whose eruption was controlled by a G.F.D. Previously, this group of workers presented evidence (Riches et al. 1973) that these vacuoles are part of the basic disease process in the skin in D.H., and the absence of such lesions in patients whose rashes disappeared following gluten withdrawal was good evidence for gluten playing a role in the pathogenesis. Fry et al. (1973) stressed that for a G.F.D. to be successful in the control of the eruption, the diet must be strict and has to be taken for many months (in one patient it took 4 years) before the rash clears. It was evident from the reports of Shuster et al. (1968) and Weinstein et al. (1971) that the diet had not been given long enough to be fully effective.

Immunological Abnormalities:

The unique association of D.H. with coeliac disease has led to complimentary immunological studies being performed in both disorders. Discussion on these immunological studies will be more enlightening when viewed in the light of both entities, and this will therefore be done throughout this thesis. Where appropriate, attempts will be made to correlate the similarities and contrasts that exist in an effort to clarify the relationship between the two disorders.

Serum Immunoglobulins

The main immunoglobulin involved in the gastro-intestinal tract is IgA (Chodirker and Tomasi 1963). With the advent of single radial micro-diffusion techniques (Mancini et al. 1965) extensive studies of immunoglobulin levels in various body fluids were performed in many diseases. In coeliac disease, attention was focused initially on serum IgA levels.

Initial reports suggested that IgA in coeliac disease may be low. Hence, Crabbé and Heremans (1966) reported 3 patients with steatorrhoea, in 2 of whom no IgA could be detected in the serum. In the third, a very low level was present. Subsequently, the same authors (Crabbé and Heremans 1967) reported that one of these patients had gluten sensitive enteropathy (G.S.E.), and suggested that IgA deficiency may be the cause of G.S.E.

Further and more extensive studies however, did not bear this out. Thus, Hobbs and Hepner (1966, 1968) found a very low incidence (less than 10%) of IgA deficiency in patients with coeliac disease, and in those who had low serum IgA levels, the deficiency was minimal. In fact, Asquith et al. (1969) reported that as a group, patients with coeliac disease had higher levels of IgA than a control series. In D.H., as a group, Fraser et al. (1969) found a similarly higher level of serum IgA, and Fry et al. (1967) found a slightly raised IgA level in one of their 12 patients.

Abnormalities of serum immunoglobulin levels were found in fact, not to be present with IgA but with serum IgM levels. Thus, a third of patients with D.H. (Fry et al. 1967, Fraser et al. 1969) and coeliac disease (Hobbs and Hepner 1968) had low levels. Hobbs and Hepner (1968) found that this deficiency was related to diet, and that on treatment with a gluten-free diet (G.F.D.) the levels returned to normal. The same group of workers, using IgM cold agglutinin iodinated with ^{125}I or ^{131}I, found that the catabolism and distribution of IgM was normal in patients with coeliac disease and suggested that the low IgM levels were due to defective

synthesis (Brown et al. 1969). They further suggest that the low serum IgM levels might be indicative of 'lymphoreticular dysfunction' which is present in coeliac disease (McCarthy et al. 1966). In support of this is the finding of splenic atrophy in both coeliac disease (Marsh and Stewart 1970) and D.H. (Pettit et al. 1972), for it is thought that the spleen is a major site of early antibody production, in particular of IgM (Rowley 1950). It is paradoxical however, that the splenic atrophy does not respond to a G.F.D., whereas the serum IgM levels do.

On the whole, no consistent pattern of serum immunoglobulin abnormality is evident in coeliac disease and D.H. Low or absent IgA levels have been found with coeliac disease, but not in D.H.

Immunoglobulins in the Small Bowel

IgA is the main immunoglobulin in the secretions of the gastro-intestinal tract (Chodiker and Tomasi 1963). In the intestinal wall, IgA producing plasma cells have been demonstrated by immunofluorescence to predominate in the normal (Crabbé and Heremans 1966). In studies of the small bowel using similar techniques, a relative deficiency of IgA producing plasma cells have been reported (Douglas et al. 1970, Soltoft 1970). These workers also reported a relative increase in the IgM cells of the small intestine. Pettingale (1971a), whilst supporting the findings of relative preponderance of IgM to IgA cells in the small intestinal mucosa in coeliac disease, found that this finding was due to a lack of IgA cells, and that the IgM cell population was in fact normal in the rectal mucosa, implying that the jejunal findings were specific to the small intestine and not generalised. They also found that as with the serum, IgM was low in the bone marrow. It is conceivable therefore, that in coeliac disease, most of the IgM producing cells have concentrated in the small bowel at the expense of other tissues.

Following on these findings, it has been postulated that coeliac disease could be due to a functional rather than a quantitative abnormality

of IgA, or IgA production in the small intestine (Beale et al. 1971). These authors attempted to put this concept to the test by giving polio and tetanus vaccine to patients with coeliac disease. Orally administered polio vaccine provokes a predominantly IgA response (Berger et al. 1967) mediated through the gastrointestinal tract. Tetanus vaccine given intramuscularly, on the other hand, stimulates a predominantly IgG response (Lawrence et al. 1965). Beale et al. (1971) gave polio vaccine to 10 patients with coeliac disease and suggested that if there was an abnormality (either quantitative or qualitative) in the small intestine in coeliac disease, this would be reflected by an impaired response manifest by low serum antibody levels. They did in fact find such impaired responses in 10 of 30 responses and possible impairment in a further 8. Tetanus responses on the other hand, were essentially normal, implying normal IgG responses. They suggested that these findings implied qualitative 'impaired' IgA responses in patients with coeliac disease, and that this could explain the local excess of IgM plasma cells and of IgM precipitating antibodies to gluten in the jejunum. The validity of these findings however, have been questioned by Pettingale (1971b), as Beale et al. (1971) did not measure the specific immunoglobulin class of the antibody to the polio virus. He suggested that the abnormal response to polio immunization may be due in fact to impaired IgM antibody synthesis as previously postulated by Brown et al. (1969).

In D.H., similar abnormalities of IgM and IgA producing plasma cells as detected by immunofluorescence have been reported by one group (Lancaster-Smith et al. 1974), but have not been substantiated.

Small Intestinal and Salivary Immunoglobulin Levels

In coeliac disease, Douglas et al. (1970) found normal IgA levels in the intestinal juice. There was however, an increase in the IgM levels.

This finding could possibly imply increased IgM production in the gut wall - a finding at variance with the impaired generalised synthesis of IgM reported by Brown et al. (1970), though consistent with the preponderance of IgM over IgA plasma cells in the submucosa (Douglas et al. 1970, Soltoft 1971).

In D.H., raised IgM levels have also been found in the small intestinal juice (Mc Clelland et al. 1972). Unlike coeliac disease, very high levels of IgA were also found, though the relevance of this is not apparent at the present time.

In the saliva, Douglas et al. (1970) found normal levels of both IgM and IgA. McClelland et al. (1972) reported marginally raised levels of IgA in the saliva in D.H., but this was not substantiated by Oon et al. (1973).

Other Immunological Abnormalities Associated with the Small Intestine

In 1964, Malik et al. demonstrated by immunofluorescence, the presence of antibodies in the sera of patients with coeliac disease, to the cytoplasm of jejunal epithelial cells. They found the incidence of these antibodies to be lower in patients on a gluten-free diet. However, these findings were not confirmed by Rubin et al. (1965).

In 1969, Dick et al. reported antibodies to the basement membrane of jejunal mucosa in 8 patients with D.H. The antibody reacted with small intestinal mucosa from normals and D.H. patients in 5 of the 8 sera, but only with D.H. mucosa in 3 sera. These findings however, were not confirmed by Holubar et al. (1971).

Immunology of the Skin in Dermatitis Herpetiformis

Cormane (1967) first reported the presence of immunoglobulin deposits in the skin in D.H. He found by immunofluorescence, immunoglobulins in both the involved and clinically uninvolved skin, but did not specifically mention the class of immunoglobulin. Subsequent studies by Jordon et al. (1967) and Chorzelski and Cormane (1968), found no circulating antibodies

to the basement membrane in patients with D.H., and no immunoglobulin deposits in the skin. However, although it was not specifically stated in either of these reports, it was implied that involved D.H. skin was studied. This is of relevance as in later studies, it was found that immunoglobulin deposits were often absent if a late blister was examined.

Van der Meer (1969) like Cormane, found immunoglobulin deposits in the clinically uninvolved skin of 10 of 12 patients in the basement membrane zone. In addition, similar deposits were found in 3 of 12 specimens of involved skin. The majority of immunoglobulin deposits were of IgA class, and in a few, IgG. He described the deposits as 'granular', but in one it appeared as a 'homogeneous' band along the basement membrane. It is of note that Van der Meer classifies his 12 patients into 5 who had 'typical' D.H. (all of whom had IgA deposits), and 7 who had 'atypical' D.H. (of whom only 5 had IgA deposits). Holubar et al. (1971) in a study of 12 patients, confirmed Van der Meer's findings. They found IgA deposits in the perilesional skin in 11 of 12 patients, but none in the actual lesions. No IgG or IgM deposits were found.

Chorzelski et al. (1971) in a similar study of D.H. skin found IgA deposits in all their 19 patients. IgG deposits were present in 6 patients and IgM deposits in 5. These authors described three patterns of immunofluorescent staining. Firstly, a 'microgranular' pattern seen in the dermal papillae; secondly, a 'fibrillar' pattern, also seen in the dermal papillae, and thirdly, a 'homogenous' pattern seen along the length of the dermo-epidermal junction. The homogenous pattern was found only with IgA, whilst IgG and IgM deposits tended to be microgranular or fibrillar. Chorzelski and his colleagues considered the homogenous pattern to be indistinguishable from that seen in pemphigoid where it is thought that the immunoglobulins are localized in the basement membrane.

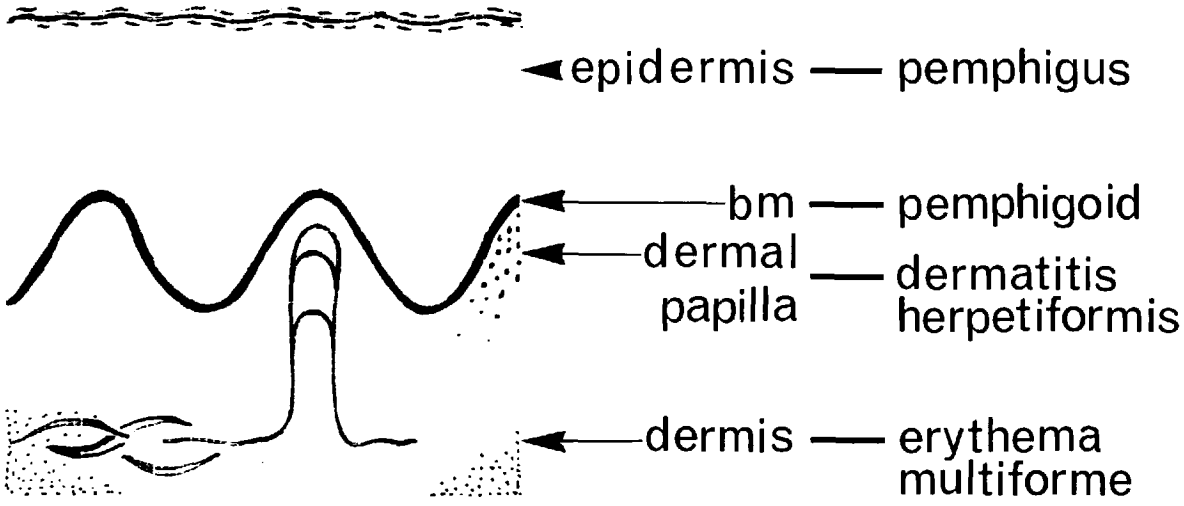


Figure I: 1

Schematic representation of the differential diagnosis of the bullous disorders at various levels in the skin at which pathological changes occur.

BM = basement membrane

PART III

PATIENTS, MATERIALS AND METHODS

Patients:

Dermatitis Herpetiformis

56 patients with D.H. were studied. These 56 comprised 32 males and 24 females. The oldest patient was born in 1895 and the youngest in 1950. At the time of writing this thesis, 5 patients for various reasons, were not being followed up and 4 patients had died. One patient (born 1921) died at the age of 50 from carcinomatosis due to a carcinoma of the bronchus. She had taken arsenical compounds for many years for the control of her D.H., and at the time of her death also had arsenical keratoses on both palms. The other three patients (all males, born in 1895, 1897 and 1897) all died from coronary thromboses. One of these also had a history of pernicious anaemia.

The diagnosis of D.H. was made on the following criteria:

1. Clinical features of the skin eruption. This usually consists of a highly irritant rash mainly affecting the pressure points - in particular, the elbows, shoulders, neck, scapulae, buttocks, and knees. The rash is frequently polymorphic as originally described by Duhring; blisters may be present, but more commonly, grouped excoriated papules on an urticarial base, or urticarial lesions only are seen.

2. Histology of skin lesions. An early erythematous lesion or blister is usually examined histologically. The diagnostic criteria of Pierard and Whimster (1961) are used. The main diagnostic features are (a) a sub-epidermal blister, (b) the presence of micro-abscesses in the dermal papillae in the surrounding erythematous skin, (c) a cellular infiltrate consisting predominantly of neutrophils and eosinophils. These histological features are illustrated in figure III, 1 a and b.

3. Clinical response to dapsone or sulphapyridine with relapse on withdrawal of therapy. Patients were initially given 100 mg of dapsone (4.4' diaminodiphenylsulphone - D.D.S.) per day, and if this was not effective,

the dose was increased to 200 mg and then to 300 mg per day. Patients were seen at weekly intervals. Nearly all patients with D.H. respond to dapsone, and on withdrawal of therapy, there is relapse of the skin rash, usually within 1 week. This therapeutic trial of dapsone is probably the most widely used criterion in the diagnosis of D.H.

Dapsone was the first drug of choice and sulphapyridine was only used in patients who could not tolerate dapsone. The dose of sulphapyridine varied from 1 to 3 grams daily.

These 3 criteria were used in the initial part of the study. None of them however, (as will be discussed later) are specific for D.H., and it soon became apparent during the course of the study that if only these criteria were used, a small number of patients will be misdiagnosed as having D.H. For this reason, two further parameters were considered in the diagnosis of D.H. These are the presence of IgA in the clinically uninvolved skin, and the presence of increased lymphocytic infiltration in the small bowel mucosa.

These two parameters will be assessed later on in this thesis and their usefullness as more specific markers in the diagnosis of D.H. evaluated.

"Non-D.H." Group

A second group of patients - the "non-D.H." group were also studied. The qualification for inclusion in this group is one or more of the following:-

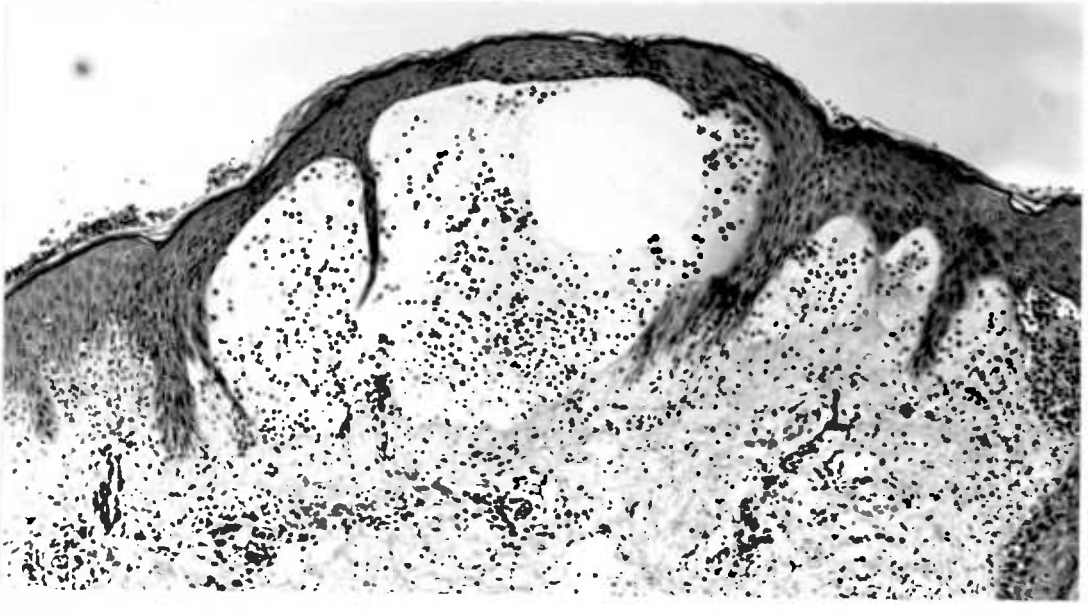
(i) Patient referred from another dermatologist with the clinical diagnosis of D.H. - not subsequently substantiated.

(ii) Patient with a rash which is clinically suggestive of D.H..

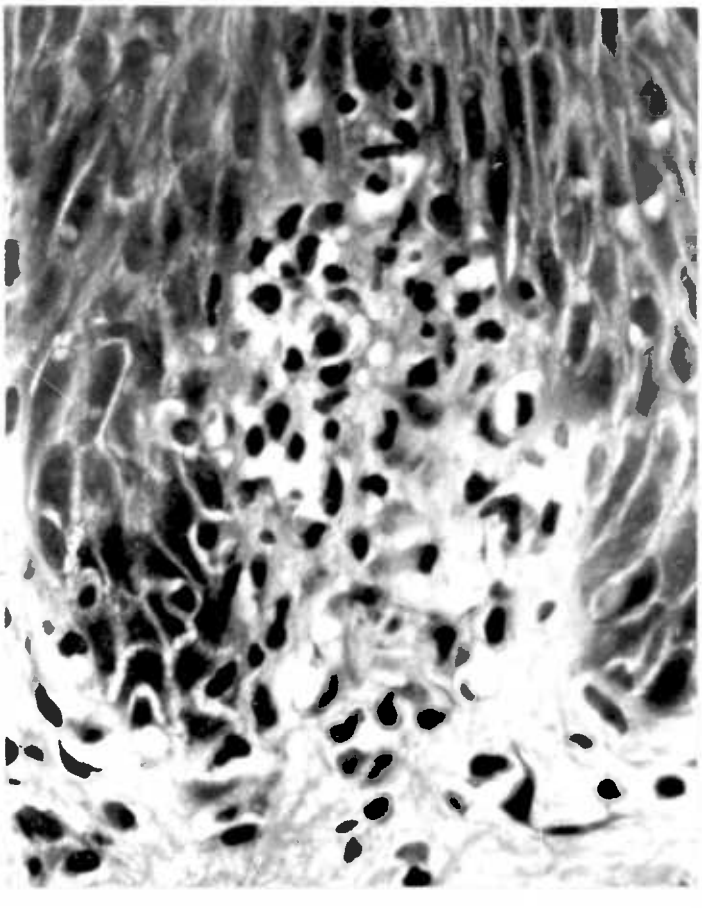
(iii) Patients with borderline or suggestive histology of a skin lesion.

(iv) Patients with dapsone or sulphapyridine responsive skin eruptions.

The majority of this group of patients were put through a similar battery of investigations as the D.H. patients in an attempt to substantiate



(a)



(b)

Figure III. 1 a and b - Histology of skin lesion in dermatitis herpetiformis

(a) low power view - showing sub-epidermal blister.

(b) high power view - showing early papillary microabscess with marked inflammatory infiltrate.

H. & E.

or refute the diagnosis of D.H.

There were 22 patients (7 males, 15 females) in this group. The oldest was born in 1904 and the youngest in 1962.

Other Patients

Data from skin patients with pemphigus, pemphigoid, vasculitis, erythema induratum, erythema multiforme and erythema nodosum who were investigated routinely in making the diagnosis, have also been included in this study as controls or for comparison.

Material from normal volunteers, and patients with various other illnesses have also been included for the same purpose.

Coeliac Disease

Material from patients with childhood and with adult coeliac disease was included in parallel with some of the studies. The diagnosis of coeliac disease (gluten-sensitive enteropathy, idiopathic steatorrhoea non-tropical sprue) was made on the finding of a macroscopically abnormal (flat or flat-with-mosaic) proximal small bowel biopsy (Booth et al. 1962), and a characteristic coeliac mucosal lesion histologically (Doniach and Shiner 1957).

The diagnoses and details of the patients and controls studied, and that of volunteers used, will be given under the specific projects where they have been involved in the appropriate sections of this thesis.

The procedures used on patients in these studies were mainly limited to three: (i) Blood collection (ii) Skin biopsy (iii) Small intestinal biopsy. Where indicated, the procedures used were fully explained to the patients and their informed consent obtained.

Skin Biopsy

Skin biopsies were performed under local anaesthesia using 1% or 2% lignocaine. Biopsies were taken either using a scalpel, or a 3 mm or 5 mm punch (John Weiss, Wigmore Street, London).

Skin biopsies were taken from the following groups:

A: Clinically involved skin.

(i) D.H. patients not on dapsone - early erythematous skin, early blisters, and/or peribullous skin.

(ii) 'Non-D.H.' group of patients.

(iii) Patients with various skin diseases, including pemphigus, pemphigoid, vasculitis, erythema induratum, erythema multiforme.

B: Clinically uninvolved skin

(i) D.H. patients on dapsone alone (controlled).

(ii) D.H. patients not on dapsone (with skin lesions i.e., relapsed).

(iii) D.H. patients on gluten-free diet only (controlled).

(iv) D.H. patients on dapsone and gluten-free diet (controlled).

(v) Coeliac disease patients.

(vi) 'Non-D.H.' group of patients.

(vii) Patients with various skin diseases, including pemphigus, pemphigoid, vasculitis, erythema multiforme, erythema nodosum.

(viii) Volunteers with no evidence of skin disease.

All the biopsies from group B were taken from the buttocks. Biopsies in group A were usually taken from the buttocks when these were involved. Otherwise, affected areas on other parts of the body were used.

Preparation for Histological Examination

Skin biopsies were mounted on white blotting paper and fixed in formol saline. They were then wax embedded, routinely processed, and stained with haematoxylin and eosin.

Preparation for Immunofluorescence

The fresh unfixed skin was placed in a cup made of aluminium foil and filled with OCT compound (details on page 39). It was then suitably orientated so that when the skin was cut, the plane of section would be at right angles to the basement membrane. The whole, (foil, skin and OCT compound) was then immersed, either in iso-pentane pre-cooled in a beaker in liquid nitrogen or directly into liquid nitrogen (Figure III.2). The specimens were then stored in a $-70^{\circ}C$ deep freeze until processed.

Procedure for Direct Immunofluorescence

4 - 6 μ sections were cut in the cryostat and transferred to glass slides. They were air dried unfixed under a forced draught for 30 minutes, then washed in phosphate buffered saline pH 7.2 (PBS) for 15 minutes and subsequently treated with fluoroscein-conjugated antiserum appropriately diluted, for 30 minutes.

In direct immunofluorescent studies where a sandwich technique (2 layers) was used, the appropriate unconjugated antiserum was first layered on for 30 minutes (1st layer). After a wash of 10 minutes in PBS, the skin sections were subsequently incubated with the appropriate fluoroscein conjugated antiserum for 30 minutes (2nd layer).

Following incubation with the fluoroscein conjugated antiserum, the skin sections were given a final wash for 1 hour in PBS and mounted in buffered glycerol pH8 (9 parts glycerol to 1 part buffer pH8). At least four sections of each skin biopsy were examined for any one particular conjugate.

Tests of Specificity of Immunoglobulin Deposits in the Skin ("Blocking" Experiments)

The aim of these experiments is to prove the specificity of immunoglobulin deposits detected in the skin biopsies using fluorescent conjugates. The specificity of an immunological reaction detected by immunofluorescence, e.g., that 'IgA' detected by FITC labelled anti-human IgA is actually IgA heavy chain, may be altered or erroneously interpreted by the following factors:-

(i) cross-reactions - the result of different antigens sharing common antigenic determinants.

(ii) valency of conjugate for multiple antigens - this may arise as a result of using impure antigens for production of the antisera. e.g., by incomplete separation of contaminants from the antigen against which a specific antiserum is being raised.

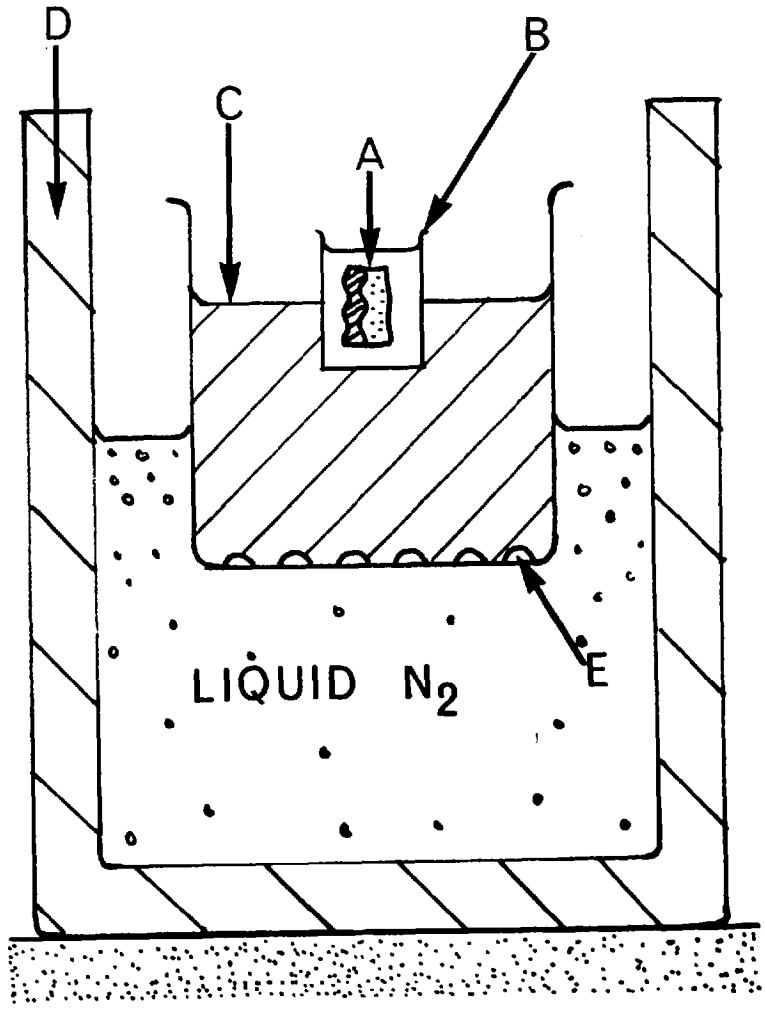


Figure III.2

Technique of snap-freezing biopsies for immunofluorescence.

The skin biopsy (A) is oriented in OCT Compound contained in a cup made of aluminium foil (average diameter 7 mm, length 1.4 cm). This is then immersed in pre-cooled isopentane (C). The isopentane is cooled in a beaker immersed in liquid nitrogen contained in a thermos flask (D). It is sufficiently cool for snap-freezing when white crystals appear on the bottom of the beaker (E). Average time for snap-freezing - 20 seconds.

(iii) free uptake of fluoroscein - either as free FITC or FITC combined with antiserum - by various components in the skin.

(iv) autofluorescence mimicking true fluorescence.

In an attempt to define the specificity of the immunoglobulins detected as being true immunological phenomenon, 'blocking' experiments have been performed. In these, unconjugated antiserum is first introduced to the antigen. Following antibody-antigen reaction, excess free antiserum is washed off, and a FITC labelled antiserum directed against the same antigen is introduced. Assuming that all the receptor sites on the antigen have been taken up by antibody from the unconjugated antiserum, there should be no further uptake by the FITC labelled antiserum. Hence, 'blocking' is effected and indicates that positive fluorescence is not due to non-specific uptake of fluoroscein, but is the result of a true antibody-antigen reaction.

Equipment:

Fluorescent Microscope

A Reichert (Austria) 'Diapan' transillumination fluorescent microscope was used throughout the study. The microscope is fitted with a 'Toric' darkfield wide angle condenser, and a low voltage halogen bulb (G.E.C. A 1/215. 12v. 100w.). Oil or glycerol is used between the slide and the condenser. For low power work, a x10 neoachromatic objective was used, and for high power, a x40 objective with a numerical aperture (N.A.) of 0.90 and adjustable iris.

A 'Balzer' Filtraflex FITC-3 primary interference filter (see figure III.3 for optical details) and a Kodak Wratten 12 secondary filter was used throughout.

Photographs were taken on Agfa C.T. 18 film (A.S.A.50 D.I.N. 18) using a Remica 3 camera with a beam splitter (100/100%) attachment.

Cryostat

A 'Bright' (Huntingdon, England) cryostat cabinet fitted with a Cambridge rocking microtome (Cambridge Scientific Instruments, England) perspex anti-roll

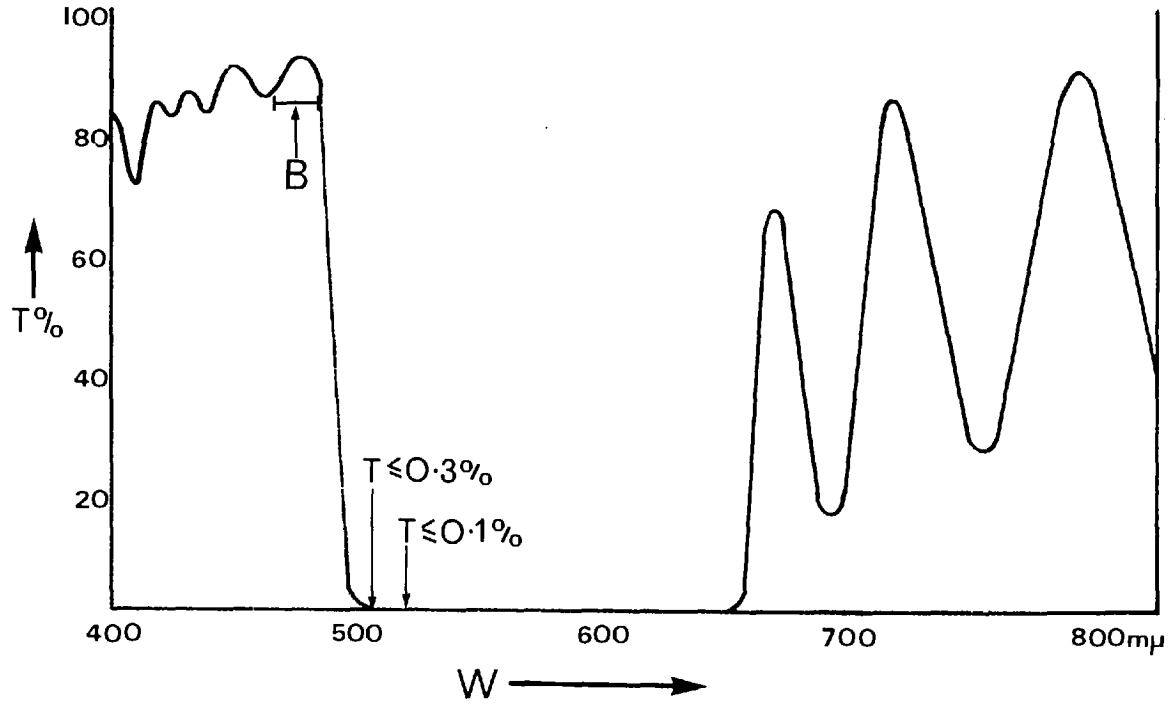


Figure III.3

Emission spectrum of Balzers Filtraflex FITC-3 primary interference filter.

The filter aims at giving nearly 100% transmission between 470mμ and 480 mμ (B on figure) with total isolation at 520 mμ.

T= % transmission; W= wavelength

Transmission
minimum 450-485 mμ.....85%

Transmission
maximum 520 mμ.....0.1%

Thickness.....0.9 - 1.1 mm

Dimension.....32 ± 0.2 mm

bar and 'Original Jung' knife was used throughout the study. It was maintained at -30°C.

Chemicals:

Phosphate Buffered Saline (PBS)

This was prepared using a commercially available compound. "FTA-Haemagglutination Buffer - pH 7.3 ± 0.2" (B.B.L.) obtained from Becton and Dickenson Ltd., Runcorn, Cheshire.

The formula in grams per litre was:

Sodium Chloride	7.65
Disodium Phosphate	1.2688
Monosodium Phosphate	0.1
Monopotassium Phosphate	0.2113

For preparing PBS, 9.23 grams of the compound was dissolved in 1 litre of distilled water.

O.C.T. Compound (Ames)

This embedding medium for frozen tissue specimens was obtained from Ames Company, Stoke Court, Stoke Poges, Slough, Bucks. O.C.T. stands for 'Optimal Cutting Temperature' and is an inert polysaccharide which is water soluble and leaves no residue or discolouration.

Gluten - Australian Vital Wheat Gluten

This was kindly provided by Energen Foods Co. Ltd., Ashford, Kent. It was stored at room temperature.

Gluten - Fraction 3

This was kindly provided by Dr. R. Schneider, Department of Clinical Pharmacology, The Medical School, University of Birmingham. It was prepared from crude gluten by peptic-tryptic digestion according to the method of Frazer et al. (1959). It was stored at -20°C. in a freeze-dried form.

Antisera, Fluoroscein Conjugates and Serological Tests:

Antisera (unconjugated)

i. Sheep-anti-human IgG Wellcome reagents MR.54 lot K.1945

ii. Sheep-anti-human IgM " " MR.55 lot K. 2121

iii. Sheep-anti-human IgA " " IP.15 lot K.6787

iv. Horse-anti-human serum " " MR.40 lot K.4898

v. Rabbit-anti-human IgG Mercia 10090 lot 072

vi. Rabbit-anti-human IgM " 10091 lot 013

vii. Rabbit-anti-human colostrum IgA Mercia 10 AT lot 023

viii. Rabbit-anti-human BIC/BIA Behringwerke AG Batch T 1865 AM

ix. Rabbit-anti-human C3 - gift of Dr. J.F. Mowbray, (Department of Experimental Pathology, St. Mary's Hospital Medical School, London.)

This was prepared by immunization of rabbits with C3 obtained by incubating fresh human serum with zymosan. The immunological specificities (and that of viii.) are as assessed by immunodiffusion illustrated in figure III.4.

xi. Rabbit-anti-human C1q - gift of Dr. J.F. Mowbray.

C1q was obtained from fresh human serum using the method of Yonemasu and Stroud (1971) and used for immunization in rabbits. The antisera obtained was then absorbed with pseudoglobulins and on immunodiffusion studies gave a single precipitin line against both C1q and fresh human serum (figure III.5.)

xii. Sheep-anti-human IgA secretory piece - gift of Dr. R.A. Thompson (Regional Immunology Laboratory, East Birmingham Hospital.)

This was produced by immunization in a sheep with IgA-secretory piece obtained from human colostrum. The antiserum was then absorbed with human serum IgA to remove any anti-7S IgA. On double immunodiffusion, no lines of precipitation were obtained against whole human serum,

Rabbit-anti human C1q
(both wells)

Fresh human
serum

Horse -anti
human serum

Rabbit-anti human C3 (B_{1A}/B_{1C})
(Behringwerke)

Rabbit-anti human C3
(Dr J F Mowbray)

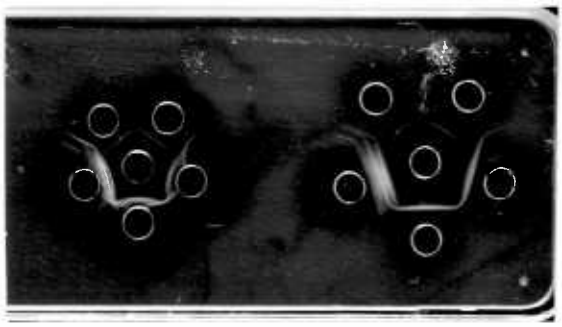


Figure III.4

Double immunodiffusion studies of two different anti-human C3 antisera against fresh human serum. In addition the specificities against anti-human C1q has been assessed. Only a single line of precipitation is obtained between the anti-C1q and fresh serum.

Figure III.5 Double immunodiffusion of sheep-anti-human IgA secretory piece against normal human serum in central well. No lines of precipitation against serum 7S IgA is seen.

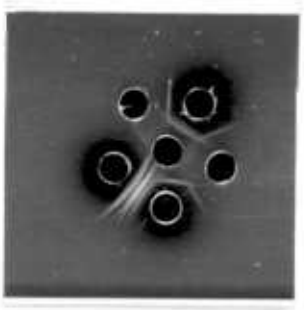


Figure III.6 Double immunodiffusion of sheep-anti human IgA secretory piece anti-serum against human jejunal juice showing lines of precipitation. The anti-serum has been absorbed with human serum to remove any anti-7S IgA - hence the lines of precipitation between the horse-anti-human serum and the anti-IgA secretory piece antisera.

and one line of precipitation obtained when tested against human jejunal juice. When tested against cow's milk no lines of precipitation were obtained.

Fluoroscein Conjugates:

(FITC = Fluoroscein iso-thiocyanate)

Technical details and specificities shown in tables.

a. FITC sheep-anti-human IgG - Wellcome Reagents (table III. 1)

b. FITC sheep-anti-human IgM - " " (table III. 2)

c. FITC sheep-anti-human IgA - " " (table III. 3)

d. FITC sheep-anti-human immunoglobulin - Wellcome Reagents (table III. 4)

e. FITC goat-anti-human C3 (B1C/B1A) - Hylands 072-2-1
(specificity assessed by testing ability of this conjugate to detect complement fixed by human IgG gastric parietal cell antibody - for details, see page 56).

f. FITC sheep-anti-rabbit immunoglobulin - Wellcome Reagents (table III. 5)

g. FITC goat-anti-rabbit immunoglobulin - Nordic

h. FITC rabbit-anti-sheep immunoglobulin - Nordic

i. FITC goat-anti-human kappa-chain (Bence-Jones-K) Nordic

j. FITC goat-anti-human Lamda-chain (Bence-Jones-L) Nordic

Serological Tests:

Thyroglobulin Antibodies: These were detected using a Wellcome Reagents Thyroglobulin Test kit, containing thyroglobulin sensitized sheep cells (preserved) and control sheep cells (preserved) prepared by the method described by Fulthorpe et al. (1961).

Detection of Autoantibodies:

Circulating autoantibodies to various human tissue components were detected by indirect immunofluorescence according to the technique of Johnson and Holborow (1973). Serum obtained from blood clotted in a plain bottle was stored at -20°C. until examined. A 1:10 dilution of the serum was initially made in phosphate

TABLE III : 1

FITC Sheep-anti-human IgG - Technical Data Wellcome Reagents (MF03)

Lot No.	K3942	K4753
Fluoroscein/protein ratio		
Optical density ratio (E495 nm : E280nm)	0.72	0.61
Molar ratio	3.2	2.6
Protein - mg/ml	9.8	9.2
Antibody protein-mg/ml	0.4	0.8
Specificity Tests		
Gel diffusion		
IgG	+	+
IgM	-	-
IgA	-	-
DIF Monoclonal		
Bone Marrow		
γ	+	+
μ	-	-
α	-	-
k	-	-
λ	-	-
Working Dilution		
Direct IF	1:32	1:32-1:60
Indirect IF	1:16-1:20	1:16-1:24

TABLE III : 2

FITC Sheep-anti-human IgM - Technical Data
Wellcome Reagents (MF04)

Lot No.	K4060	K4392	K6762
Fluoroscein/protein ratio			
Optical density ratio ($E_{495\ nm} : E_{280\ nm}$)	0.66	0.65	0.68
Molar ratio	3.0	2.6	3.0
Protein - mg/ml	5.8	4.0	5.5
Specificity Tests			
Gel diffusion			
IgG	-	-	-
IgM	+	+	+
IgA	-	-	-
DIF Monoclonal			
Bone Marrow			
γ	-	-	-
μ	+	+	+
α	-	-	-
k	-	-	-
λ	-	-	ND
Working Dilution			
Direct IF	1:12-1:24	1:12-1:24	1:12-1:24
Indirect IF	1:24	1:12-1:24	1:12-1:24

ND = not done

TABLE III : 3

FITC Sheep-anti-human IgA - Technical Data
Wellcome Reagents (MF05)

Lot No.	K3914	K4293	K5777	K7307
Fluoroscein/protein ratio				
Optical density ratio ($E_{495\ nm} : E_{280\ nm}$)	0.57	0.58	0.71	0.85
Molar ratio	2.4	2.4	3.3	4.1
Protein - mg/ml	7.0	5.9	2.9	5.0
Specificity Tests				
Gel Diffusion				
IgG	-	-	-	-
IgM	-	-	-	-
IgA	+	+	+	+
DIF Monoclonal				
Bone Marrow				
γ	-	-	-	-
μ	-	-	-	-
α	+	+	+	+
k	-	-	-	-
λ	-	-	ND	ND
Working Dilution				
Direct IF	1:8-1:12	1:8-1:12	1:8	1:8-1:12
Indirect IF	1:12	1:12	1:12	1:12

ND = not done

TABLE III : 4

FITC Sheep-anti-human Immunoglobulin - Technical Data
Wellcome Reagents (MF01)

Lot No.	K3713
Fluoroscein/protein ratio	
Opitcal density ratio ($E_{495\ nm} : E_{280\ nm}$)	0.85
Protein - mg/ml	8.6
Antibody protein (anti-IgG) - mg/ml	1.1
Gel Diffusion Tests	
IgG	+
IgM	+
IgA	+
Working Dilution	
for indirect IF	1:32

TABLE III : 5

FITC Sheep-anti-Rabbit Immunoglobulin - Technical Data
Wellcome Reagents (MF02)

Lot No.	K5865	K6309
Fluoroscein/protein ratio		
Optical density ratio ($E_{495\ nm} : E_{280\ nm}$)	0.8	0.8
Molar ratio	3.7	3.7
Antibody protein-mg/ml	1.15	1.15
Protein (Biuret)-mg/ml	9.7	9.7
Working Dilutions		
Direct IF	1:5-1:10	1:5-1:10
Indirect IF	1:16	1:16

buffered saline (PBS) pH 7.2 - 7.4. For titration purposes, stepwise dilution (1:20, 1:40, 1:80, 1:160 etc.) was done. All sera were screened at the initial 1:10 dilution. Neat serum was not used. Generally, the sera were not heat inactivated (for complement), but where necessary, the serum was incubated in a water bath at 56°C. for 30 minutes.

The following autoantibodies were looked for -

1. Anti-nuclear antibody (ANA)
2. Smooth Muscle antibody (SMA)
3. Gastric parietal cell antibody (GPC)
4. Mitochondrial antibody (Mito.)
5. Thyroid microsomal antibody.
6. Thyroglobulin antibody.

For (1) to (4), the source of antigen was cryostat sections of rat liver (for ANA - Holborow et al. 1957), rat stomach (for GPC - Irvine et al. 1962, and for SMA - Johnson et al. 1966), and rat kidney (for mito. - Walker et al. 1965). These three tissues were obtained from a freshly killed 'Black Hood' rat (less than 3 months old). A wedge of liver, kidney (cortex + medulla) and stomach (pyloric end) were arranged on a cryostat chuck and immediately snap-frozen on a carbon-dioxide snow freezing device (Slee, England). This 'composite block', when cut in the cryostat, gave sections of about 1 cm. diameter containing all three tissues (see figure III. 7) necessary for detecting ANA, SMA, GPC and Mito. 6 μ sections were cut and transferred on to glass slides. After drying in a forced draught for 15 to 20 minutes, the sections were incubated with the 1:10 dilution of serum and left in a moist chamber for 30 minutes. They were then washed for 15 minutes in PBS using a magnetic stirrer (Gallenkemp) in a water bath. After removal of excess buffer with tissue paper, the sections were incubated with the appropriately diluted fluoroscein-conjugated antiserum for 30 minutes, again in a moist chamber. The sections were then given a final wash of 1 hour in PBS, and

subsequently mounted in buffered glycerol pH8 (9 parts glycerol to 1 part buffer pH8). During the whole procedure (other than the initial drying in a forced draught) the sections were never allowed to dry out completely, as this gave rise to artefacts.

The sections were examined either immediately, or within 24 hours. For the latter, they were stored at +4°C.

A similar procedure was empolyed for the detection of thyroid microsomal antibody. As a source of antigen, frozen human thyroid (removed from a patient with thyrotoxicosis) 6 µ was used (Holborow et al. 1959).

For all these tests, known positive and negative sera were also included as controls in every batch.

Thyroglobulin antibody was detected using a Wellcome Thyroglobulin Haemagglutination Test Kit, accoring to the method of Fulthorpe et al. (1961). Perspex agglutination trays (Chromic acid washed and grease free) were used.

The following modified procedure was used.

1. Absorption of patient's serum to remove antibody to sheep red cells (found in normals) was performed by incubation of 0.1 ml test serum with 0.4 ml preserved control sheep red blood cells (making 1:5 dilution). After standing at room temperature for 10 minutes, the mixture was centrifuged and the supernatant used in the test. For dilution, PBS pH 7.3 with 0.15% foetal calf serum was used.

2. The dilutions were made in the following manner.

Set up:-

Well no.	Diluent	Serum
1	-	0.1 ml of 1/5 dilution
2	0.4 ml	0.1 ml of 1/5 dilution
3	0.4 ml	-
4	0.4 ml	-
5	0.4 ml	-
6	0.4 ml	-
7	0.4 ml	-
8	-	0.1 ml of 1/5 dilution

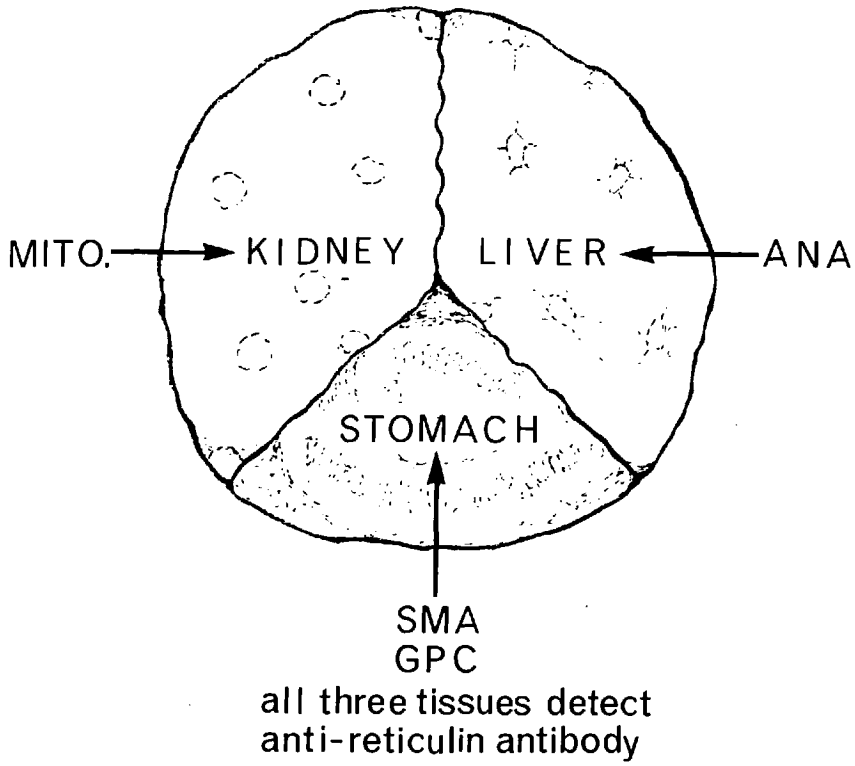


Figure III. 7

Diagramatic representation of composite block made up of rat liver, stomach (pyloric end) and kidney (cortex and medulla) used for detection of auto-antibodies. All three tissues will detect anti-reticulin antibody. The stomach detects gastric parietal cell (GPC) and smooth muscle (SMA) antibodies; the liver, anti-nuclear antibodies (ANA) and the kidney, mitochondrial (mito) antibodies.

3. Mix Well 2 and transfer 0.1 ml from Well 2 to 3. Mix and transfer 0.1 ml from 3 to 4 and so on till Well 7. Discard the last 0.1 ml.

4. Starting from the lowest dilution (Well 7) and using an automatic pipette, remove 0.3 ml from each Well, leaving 0.1 ml.

5. Mix by gently rotating the glass tray and then leave in moist chamber overnight (approximate results can be obtained at 3 hours).

Using this technique, the following dilutions are obtained:

Well No.	1	2	3	4	5	6	7	8
Dilution	$^1/_5$	$^1/_{25}$	$^1/_{125}$	$^1/_{625}$	$^1/_{3,125}$	$^1/_{15,625}$	$^1/_{78,125}$	Control

Reading the test.

The test is positive when the cells agglutinate and settle to the bottom of the cup as a diffuse carpet (this is facilitated by addition of protein - as foetal calf serum - to the diluent). A negative test appears as a compact button of cells. Weakly positive results give intermediate patterns. The end point is taken as the highest dilution giving a clear positive test; weak positives are ignored. The control must always be negative. If agglutination is seen, absorption of the test serum with control sheep red blood cells must be repeated. Known positive sera (from patients with Hashimoto's thyroiditis) were included in every batch as positive controls.

Scoring System used in Assessing Smooth Muscle Antibody (SMA).

The presence of SMA was assessed by the system introduced by Whitehouse and Holborow (1971). In the rat composite block used in this system SMA stains (i) fibres between the gastric glands, in the muscularis mucosae and the walls of arteries in the stomach, (ii) the area around liver cells (polygonal staining) - (Johnson et al. 1966), (iii) and in the kidney, the renal glomeruli (Whittingham et al. 1966). All these five sites in rat tissue contain antigenic determinants in common with human smooth muscle (Farrow et al. 1971).

Each of the five features described above was scored as follows:-

0 = no staining
1 = probable staining
2 = definite staining
3 = strong staining

The total score for each test serum was therefore assessed out of a possible maximum of 15. 0 - 5 was regarded as negative, 6 - 9 as weak positive and 10 - 15 as strong positive.

Detection of Pemphigus and Pemphigoid Autoantibodies.

Circulating antibodies to intercellular cement substance (as found in pemphigus) and to basement membrane (as found in pemphigoid) were detected as described by Beutner et al. (1970) using guinea pig lip and oesophagus. A polyvalent FITC labelled sheep-anti-human immunoglobulin conjugate (Wellcome) was used at a dilution of 1 : 32.

Detection of Anti-reticulin Antibody (ARA)

ARA was detected using the rat composite block previously described. Connective tissue in all three tissues, i.e., liver, stomach and kidney act as sources of antigen in the indirect immunofluorescence test used to detect ARA. The indirect immunofluorescent test used was the same as previously described for detecting circulating antoantibodies. All sera were tested at an initial dilution of 1 : 10. For titration, stepwise dilutions of 1 : 20, 1 : 40 and 1 : 80 were made when the screening test was positive. The end point of the titration was taken when a weak but definitely positive result was obtained. All sera were tested blind without knowledge of name or the diagnosis of the patient. In each test batch, known positive and negative controls were included for comparison. Sera were tested using FITC-labelled mono-specific anti-human IgG, IgM or IgA, or anti-whole human immunoglobulin (see tables III. 1 - 4).

Patterns of Staining obtained with Anti-reticulin Antibody (ARA)

The patterns of fluorescent staining obtained with ARA positive sera were assessed by paying attention to the parts of the connective tissue in

the rat composite block taking up the fluorescent conjugate. The areas of these tissues assessed were:-

1. In the liver - i. Portal tracts.
   ii. Centrilobular vein.
   iii. Perivascular connective tissue.
   iv. Inter-hepatocytes.
   v. Liver parenchyma.
   vi. Liver sinusoids.

2. In the stomach- i. Inter-gastric gland connective tissue.
   ii. Sub-mucosa.
   iii. Connective tissue surrounding smooth muscle fibres.

3. In the kidney - i. Peri-vascular connective tissue.
   ii. Peri-tubular connective tissue.
   iii. Glomerulus.
   iv. Bowman's capsule.

The patterns of stainging obtained were correlated with the clinical diagnosis of the patient.

Correlation of ARA Fluorescent Staining Patterns with Histological Stains for Connective Tissue Components.

Parallel 6/u sections of rat composite block were cut and subsequently fixed and appropriately stained for reticulin and collagen. The histological patterns obtained were then compared with those produced by indirect immunofluorescent staining seen with ARA positive sera.

The following stains were done:

1. Haematoxylin and eosin.
2. Gomori silver stain for reticulin (Gomori 1937).
3. Van Giesen stain for collagen (9 parts of saturated aqueous picric acid to 1 part of 1% acid fuchsin).

Effect of Collagenase on ARA Staining and Histological Staining.

This experiment aims at defining the effect of collagenase on the antigenic determinants of connective tissue reactive with ARA positive sera.

Materials.

6/u sections of rat composite block.

Collagenase type III Fraction A - (Sigma London Chemical Co.) obtained from clostridium histolyticum - subsequently chromatographically purified and substantially free of peptidase and trypsin-like activity.

TRIS Buffer - containing 0.05M TRIS with 0.005M calcium chloride to pH 7.0 with HCl.

2 ARA positive sera (IgG class) from a patient with D.H. and a patient with adult coeliac disease.

1 ARA negative (normal) serum.

Procedure.

After air drying, the sections of rat tissues were treated with collagenase at a concentration of 0.5 mgm and 1mgm per ml in buffer. Experiments were done in triplicate - one slide was used for immunofluorescent staining, one for staining for collagen (van Giesen) and the last for staining for reticulin (Gomori).

After treatment with collagenase (volume - 1 drop of Pasteur pipette), the slides were incubated for the following periods of time in a damp chamber at 37°C: 30 minutes, 1 hour, 2 hours, 3 hours, 18 hours. At the end of each period of incubation the collagenase was removed and the slide washed for 5 minutes in a water bath filled with PBS pH 7.2. Subsequently, the slide for immunofluorescent staining was treated with a 1 : 5 dilution of the ARA positive sera for 30 minutes. After a wash for 10 minutes in PBS pH 7.2 they were treated with FITC anti-human IgG for 30 minutes and then given a final wash of 1 hour before mounting in buffered glycerol. The slides for histological stains were fixed in formol saline after washing, and then appropriately stained for collagen and reticulin.

Controls: These were provided by -

i. Untreated sections of rat tissue.

ii. Sections treated for the same periods of time with buffer only and then stained with ARA positive and negative sera.

iii. Collagenase treated sections (½ hour to 18 hours) stained with ARA negative serum.

Collagenase Form I (Sigma) was used initially in this study, but proved to be unsuitable as the higher content of peptidases and trypsin in this less purified preparation caused considerable digestion of the cryostat sections with gross distortion of the normal architecture of the tissues.

Immunoperoxidase Staining and Immunoelectromicroscopy using ARA positive Serum.

This was kindly performed by Mrs. Janet Webb and Mr. Jack Dorling, M.R.C. Rheumatism Unit, Taplow, Berkshire.

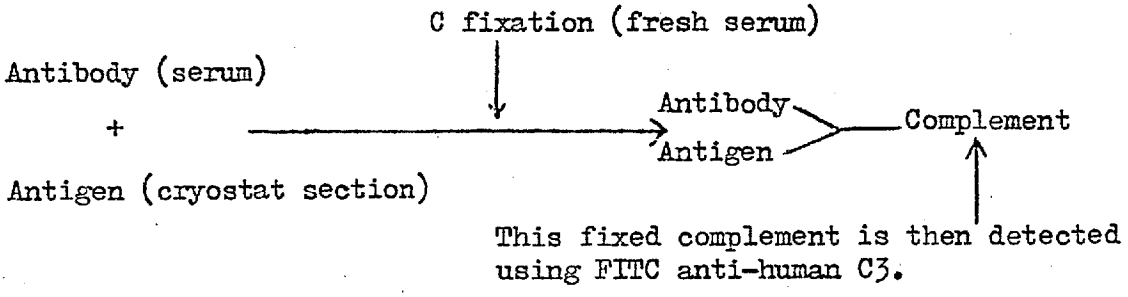
The method was that of Webb and Dorling (1973). Fresh rat liver was fixed in 1% paraformaldehyde in 0.1M phosphate buffer at pH 7.2 - 7.4 containing 7.5% sucrose for 30 minutes at 4°C. Cryostat sections were then cut and treated with an ARA (IgG class) positive serum (dilution 1 : 10) from a patient with adult coeliac disease, then with horse-radish peroxidase sheep-anti-human IgG, and subsequently processed histochemically to localize the site of the antigen-antibody reaction, as described by Webb and Dorling (1973).

The sections were viewed under light microscopy at this stage and photographed. Suitable specimens were then set in araldite and processed for immunoelectronmicroscopy.

Testing for ability of anti-reticulin antibody to fix complement in vitro.

This was done by indirect immunofluorescence. The ability of ARA to fix complement is tested by incubating ARA positive serum diluted with fresh serum (as a source of complement) with cryostat sections of rat composite block: The presence of fixed C3 is subsequently looked for by staining with FITC-anti-human C3.

The principle of this test is shown schematically as follows:-



Patients. 12 ARA positive serum, all of which contained IgG ARA's from 4 patients with D.H., 4 patients with childhool coeliac disease and 4 patients with adult coeliac disease were used in this experiment. All the test sera were complement deactivated by incubation at 56°C. for 30 minutes.

Source of complement. This was provided from fresh serum obtained from clotted blood from a normal volunteer. A portion of this was complement deactivated and used in the control experiments.

Controls. Positive control - Gastric parietal cell antibody positive serum of IgG class from a patient with pernicious anaemia was used at a dilution of 1 : 10 and 1 : 200 (dilutions made with normal serum diluted with an equal volume of PBS pH 7.3). IgG gastric parietal cell antibody is known to be strongly complement fixing. The negative control was provided by making the dilutions up with complement deactivated normal serum. The point of doing this negative control was to test the specificity of the FITC anti-human C3 for activated complement. The sensitivity of this test of specificity was enhanced by diluting the positive serum to 1 : 200. The 12 test sera were diluted 1 : 10 with both fresh and deactivated serum-buffer mixture and incubated with cryostat sections of rat composite block for 30 minutes. They were then washed for 10 minutes in PBS and subsequently incubated with a 1/10 dilution of FITC anti-human C3 (Hylands) for 30 minutes. They were then washed and mounted in the usual way. The positive and negative controls (GPC positive serum) were treated in the same way.

The presence of ARA or GPC antibody in the sera used was confirmed by staining similarly treated sections in parallel with FITC anti-human IgG instead of FITC anti-human C3.

Absorption Studies:

Absorption of ARA positive sera from patients with gluten sensitivity with gluten.

17 ARA positive sera, randomly selected from a known batch of sera from patients with D.H. and with childhood (C.C.D.) or adult coeliac disease (A.C.D.)

were used in this study. The patients were 7 with C.C.D., 4 with A.C.D. and 6 with D.H. They had IgG, IgA or both IgG and IgA ARA activity. All these sera were absorbed with (i) crude gluten (mainly insoluble)
(ii) Gluten-Fraction 3 (completely soluble)

Absorption Procedure.

1 : 10 dilutions of all the positive ARA sera were made in PBS pH 7.3. Separate 0.5 ml aliquots of the diluted serum were absorbed with 5 and 50 mg respectively of crude gluten and identically with lyophilised gluten-fraction 3. The mixtures were incubated for 1 hour at room temperature with agitation. Specimens were then spun down for 30 minutes at 3,000 r.p.m. (800g) and the supernatants removed for testing for ARA activity by immunofluorescence. Complete separation of crude-gluten by centrifugation was very difficult as the substance is insoluble and very flocculant. Spinning at higher speeds (up to 8,000 r.p.m.5240g) was equally ineffective. The remaining absorption mixtures were then incubated at +4°C. for a further 24 hours, and further aliquots removed for testing for ARA. To minimize experimental variations, all absorptions and fluorescent staining were performed in one sitting. In addition tissue from the same rat was used throughout.

Controls.

Positive controls. These were provided by the appropriate unabsorbed (1:10) serum. They were stained in parallel with the absorbed aliquots so that direct comparison of the intensity and pattern of immunofluorescent staining could be assessed.

Other controls. To assess the possibility of non-specific effects of gluten and gluten-fraction 3 on autoantibodies and immunoglobulins, positive sera from the following patients were also put through the same absorption procedures:-

1. Antinuclear antibody positive serum from a patient with systemic lupus erythematosus (IgG and IgM class).
2. Smooth muscle antibody positive serum from a patient with chronic active hepatitis (IgG class).

3. Gastric parietal cell antibody positive serum from a patient with pernicious anaemia (IgG class).
4. Mitochondrial antibody positive serum from a patient with primary biliary cirrhosis (IgG class).

In the immunofluorescent staining, FITC sheep-anti-human immunoglobulin was used in a dilution where no non-specific staining was seen.

The effect of gluten-fraction 3 on the pH of the buffer was determined:

at a concentration of 5 mg Fraction 3 in 0.5 ml PBS pH 7.3 the pH was 6.9

at a concentration of 50 mg Fraction 3 in 0.5 ml PBS pH 7.3 the pH was 6.1

Immunofluorescent Staining with Experimental anti-Connective Tissue Antisera.

Immunofluorescent staining of rat composite block with various experimentally produced anti-connective tissue antisera was performed for comparison with the pattern of staining obtained with ARA positive sera from patients with gluten-sensitive enteropathy. The chemical composition of the antigens used in producing these antisera were fairly well defined and it was therefore relevant to compare the staining patterns produced by antisera to them in attempting to clarify and define the nature of the antigen(s) involved in the production of ARA in gluten-sensitivity patients. The following experimental antisera were examined:-

1. Rabbit anti-bovine heart valves (BHV) - gift of Dr. E. Kasp-Grochowska, M.R.C. Rheumatism Unit, Taplow, Berks. This antiserum was produced as described by Kasp-Grochowska et al. (1972). The antigen was a homogenate of whole bovine valves as recommended by Goldstein et al. (1967).

Using the fractionation procedure of Goldstein et al. (1967), two components of the bovine heart valve were obtained and used subsequently in absorption procedures with ARA positive sera. These components (as described by Kasp-Grochowska et al. 1972) are:-

i. Crude Soluble Collagen (CSC)
ii. Calcium Chloride - TRIS - Citric Acid Buffer extract (CTC)

The chemical composition of these extracts are:-

	Hydroxyproline	Hexoses as Galactose	Hexosamines as glucosamine
CSC	2.0 - 2.4%	3.1 - 3.8%	2.1 - 2.5%
CTC	0.1 - 0.3%	4.7 - 4.9%	3.7 - 5.6%

	Hexuronic Acid as glucuronic	Reducing sugars as galactose
CSC	3.7%	11.5%
CTC	5.9%	20.0%

2. Rabbit anti-saline-insoluble non-collagenous reticulin component (NCRC) of pig (and human)kidney. - Gift of Dr. M. Pras, Kennedy Institute of Rheumatology, London. The NCRC was prepared and isolated by water dispersion from human and porcine organs as described by Pras and Glynn (1973). The chemical composition and amino-acid sequence of porcine NCRC had previously been defined by Pras and Glynn (1973). NCRC of both porcine and human origins were used for absorption procedures with ARA positive sera from patients with gluten-sensitive enteropathy. The antiserum was produced by immunization of NCRC in rabbits as described by Pras et al. (1974). Rabbits were given 1 ml of an emulsion containing 1 mgm of NCRC in complete Freund's adjuvant subcutaneously in five sites. The animals were bled after four weeks, boosted after six weeks and bled again ten days after the second injection.

Absorption Procedures with Connective Tissue Extracts.

1. Bovine Heart Valve (BHV) Extract.

Absorptions were performed using the Crude Soluble Collagen (CSC) and CTC Extracts - as described on page 59.

Absorption Procedures.

Absorption with CSC.

(1) 2 ARA positive sera (from 1 D.H. patient and one adult coeliac patient) were used in this study. The ARA positive serum from the coeliac disease patient also had smooth muscle antibody of high titre - and this served as an internal control in the test system.

(2) The test sera were diluted to make 1:2, 1:4, 1:8, 1:16 and 1:32 dilutions in normal saline.

(3) The diluted sera were then divided into (a) control dilutions - unabsorbed and (b) test dilutions for absorption. The test dilutions were then absorbed with CSC 1 mgm/0.1 ml of diluted serum (i.e., 10 mgm/ml) for 24 hours at +4°C with occasional agitation.

(4) The absorption mixture was then centrifuged at 2,500 rpm for 20 minutes and the supernatants further absorbed at a concentration of 0.1 mgm CSC per 0.1 ml diluted test serum for a further 12 hours at +4°C. The final absorption mixtures were then centrifuged for 20 mintues at 2,500 rpm and the supernatants removed for testing on rat composite block sections.

(5) The control serum dilutions were left at +4°C throughout. However, they were also put through the two centrifugation procedures. In the immunofluorescent procedure, the appropriate test and control sera were tested on sections placed immediately next to each other, so that a direct comparison was possible without the need to change the slides on the fluorescent microscope. This avoids variation from microscope objective and condenser adjustments.

Absorption with CTC Extract.

A similar absorption procedure was performed using the more soluble CTC extract. Two D.H. ARA positive sera were used in this experiment. In addition, absorption with CSC was performed at the same time.

2 mgm of CTC or CSC were added to
0.5 mls of 1:5, 1:10, 1:20 and 1:40 dilutions of the ARA positive serum in normal saline: Unabsorbed aliquots of the same serum dilutions served as controls.

The absorption mixtures and controls were continuously agitated at room temperature for 1 hour and then left at +4°C for 24 hours. After centrifuging at 2,500 rpm for 20 minutes, the supernatants were removed and tested as before for ARA.

2. Non-Collagenous Reticulin Component (NCRC) of Normal Human Kidney.

11 ARA positive sera from 7 patients with coeliac disease, and 4 patients with D.H. were used in this study. Sera positive for antinuclear antibody and smooth muscle, gastric parietal cell and mitochondrial antibodies were used as controls. Sera were incubated with the saline insoluble NCRC for 1 hour at 37°C. The mixture was then centrifuged in a Spinco 40 rotor at 15,000 rpm for 45 minutes. A high speed was ineffective for separation as there was a tendency for the NCRC to re-disperse in the presence of serum. The supernatants were then removed and tested for the presence of ARA. The parallel unabsorbed diluted ARA sera (which was also incubated and centrifuged) were used as the positive controls against which the degree of absorption effected could be assessed.

Histocompatibility Typing Studies:

Patients. 39 patients with D.H. and 36 patients with adult coeliac disease (ACD) were included in this study. All of the ACD patients and 38 of the D.H. patients were Caucasians; one D.H. patient was an Egyptian Arab.

Controls. These were provided from the Tissue Typing Laboratory at St. Mary's Hospital, London, W.2. Data from 100 individuals, mainly Caucasian, comprising pregnant women, blood donors and laboratory staff were used. For the 4a and 4b antigens of Van Rood, data from 923 similar controls were used.

Histocompatibility Typing.

Lymphocytes from defibrinated blood collected into clean Universal bottles were used in a modified two-stage Kissmeyer-Neilsen lymphocyte microcytotoxicity test. Lymphocytes were isolated by mixing 2 mls of defibrinated blood mixed with an equal volume of diluent (Complement Fixation Test - CFT - diluent tablets, pH 7.2 - Oxoid) and carefully layered on to a 3 ml Triocil/Ficoll density gradient (6 mls 9% solution of Ficoll + 2.5 mls of 34% solution of Triocil) in a 10 ml tube. The tube was centrifuged for 15 minutes at 3,000 rpm at room temperature. The lymphocytes sediment to

the interface during centrifugation and were carefully pipetted off. Almost all the erythrocytes and granulocytes settle to the bottom of the tube.

The cell suspension was then washed with large volumes of CFT diluent and spun down at room temperature. The procedure was repeated again, and the lymphocytes resuspended in fresh normal serum 1 : 1 parts rabbit to human serum. A cell count was done and the concentration of lymphocytes adjusted to 1 x 10^6 per cm^3.

For the typing, 1 μl of the different typing antisera were placed in the microchambers of a Terasaki plate. 1 μl of the test lymphocyte suspension was then added to each chamber using a Hamilton syringe. The plate was then incubated at 37°C for 30 minutes. 1 μl of a complement source (1 : 1 parts normal human to rabbit serum) was then added to each microchamber and further incubated for 30 minutes at 37°C. Subsequently, 1 μl of 1% trypan blue was added, and the plate read immediately for evidence of lymphocyte cytotoxicity (as shown by uptake of trypan blue by dead cells) on an inverted phase contrast microscope.

Specificities Tested.

The specificities tested were the 1st and 2nd segregant series of the HL-A system, and the 4a and 4b complex of antigens of Van Rood. The relationship of the 4a and 4b complex to the HL-A system remains uncertain at the present time. It was decided to test for these antigens in view of the frequent association of 4b with the HL-A 1, 8 chromosome. An increased incidence of HL-A 1 and 8 had previously been reported in patients with ACD (Stokes et al. 1972, Falchuk et al. 1972) and with D.H. (Katz et al. 1972, White et al. 1973, Gebhard et al. 1973).

In the HL-A system, the following specificities were tested for in the 1st and 2nd segregant series: HL-A 1, 2, 3, 9, 10, 11, W28, Da25, W32; HL-A 5, 8, 12, 13, W5, W10, W14, W15, W16, W17, W18, W21, W22, W27 and W20. Antisera to each of the histocompatibility antigens varied from 1 to 17 different sera and were obtained from multiple pregnancy and sensitized kidney transplant patients. The antisera used for typing were a mixture

obtained from patients at St. Mary's Hospital, Paddington, London, and those obtained from a number of typing centres. The specificities have been validated by parallel typing with antisera from the National Tissue Typing Reference Laboratory and London Hospital plates.

The 4a and 4b antisera were in part a gift from Professor J.J. Van Rood (Leiden, Netherlands) and some locally obtained sera which consistently gave similar reactions to these.

Relationship to Small Intestinal Biopsies.

The relationship of the HL-A antigens to the small intestinal biopsies in the D.H. patients was assessed. The procedure for obtaining and evaluating these biopsies is described below.

Small Intestinal Biopsy.

Biopsy Procedure

This was performed per-orally using the Crosby Capsule (Crosby and Kugler 1957). The capsule used in this study was of stainless steel, measuring 18.5 mm in length, 9.5 mm in diameter with a side opening or port of 3.6 mm diameter. Polythene Portex "53" tubing, or radio-opaque catheter tubing (Kifa, Sweden, Catalogue number 17.887-1) was attached to the capsule and used for firing the knife. Firing was effected by suction using a 20 ml syringe.

Intestinal biopsies were performed in the morning and patients were starved from the night before. No premedication or sedation was used as a rule. Most subjects had no difficulty in swallowing the capsule, a few sips of water being given if necessary.

Small intestinal biopsies were done under X-ray control. Two modifications of the technique of biopsy with the Crosby capsule were used. In the first, a second polythene tube was attached alongside the main tube using waterproof sleek (or 'Micropore' tape) as described by Fry (1964). This tube serves two purposes. Firstly, it avoids fluoroscopy, for fluid can be aspirated and its pH determined with universal indicator paper. The

pH and nature of the fluid aspirated gives a good indication of the position of the capsule. Hence, a pH of 7 or more indicates that small intestinal juice is being aspirated and that the capsule has passed through the pylorus. Thick, 'oily' yellow bile stained fluid also indicates that the capsule has passed the pylorus. The position of the capsule can then be clarified by injecting "Gastrograffin" or "Hypaque" into the catheter and taking a plain X-ray of the abdomen. Hence, fluoroscopy is avoided and no radiologist is required. The other advantage of the second catheter is that intestinal juice can be obtained for analysis during the same procedure.

The second modification employed is that of using a stiff outer polythene cuff to guide the capsule into position under X-ray screening, as described by Evans et al. (1970). The advantage of this procedure is that it is quick and causes minimum discomfort to the patient.

Biopsies were then taken from the 4th part of the duodenum or the duodeno-jejunal flexure after verification of the position of the capsule by X-ray. Specimens were mounted on to white blotting paper and fixed in formol saline.

Macroscopic Appearance

The mascroscopic appearance of the small intestinal biopsy was assessed using a Zeiss Standard Universal microscope with a 3" objective. Specimens were photographed through a camera attachment for record purposes.

The terminology used for classifying the macroscopic appearances of the small intestinal biopsy is that employed by Booth et al. (1962). Thus, the mucosal surface is described as flat (or flat mosaic), convoluted (or convoluted mosaic) or having leaf-like (leaves) or finger-like (fingers) villi. The flat mucosa under the dissecting microscope tends to correspond to 'subtotal villous atrophy' and the convoluted mucosal surface to 'partial villous atrophy' as originally described histologically by Shiner and Doniach (1960). 'Flat' and 'convoluted' specimens were considered to be abnormal,

whilst 'leaves' and 'fingers' were normal in this study. (Figures III. 8 to 11).

Microscopic Appearance (Figures III. 12 to 14)

Following assessment for the macroscopic appearance, embedding of the intestinal biopsy in paraffin was carried out in the usual manner. 7μ sections were cut and stained with haematoxylin and eosin or with iron haematoxylin and picrofuchsin (Van Gieson) for routine histological examination.

Quantitation of intraepithelial lymphocytes was performed as an index of cellular infiltration of the small intestine. Intraepithelial lymphocytes and epithelial cells were counted in parts of the specimens where the plane of section was vertical through the epithelium and the underlying basement membrane. The number of lymphocytes among 1000 epithelial cells were counted in each specimen and the results expressed as lymphocytes per 1000 epithelial cells. In villous specimens, counts were made on the sides and tips of the villi and in convoluted and flat specimens, the surface cells were counted. No attention was paid to crypt cells which in both control and gluten sensitive enteropathy specimens show very little cellular infiltration compared with villous cells. Low power magnification usually defines easily the transition between crypt and villus and counts were kept above this level.

Epithelial cells and lymphocytes were easily identified by their nuclei which are characteristically different. The columnar cell nuclei are pale staining and more oval or elongated than those of lymphocytes which are smaller, round and stain more darkly.

Positioning of intra-epithelial lymphocytes.

The position of the lymphocytes in relation to the epithelial cell nucleus was also assessed and classified into three categories - basal, perinuclear and supranuclear (see figure III. 15). Lymphocytes were recorded as being basal if their nuclei lay adjacent to the epithelial cell connective tissue junction. They were considered to be perinuclear if they lay above this basal position and were clustered around the epithelial cell nuclei, and supranuclear if they were entirely above the general level of the uppermost

edge of the epithelial nuclei. In properly orientated sections, there was no difficulty in assigning the lymphocytes to one of these categories. In random sections of any biopsy however, there are usually a number of areas which are oblique, and these are unsuitable for counting. In addition, it is not always possible to count 1000 epithelial cells in continuity up and down the contiguous sides of adjacent villi, and the total count has to be made usually from varying lengths of epithelium from different parts of the specimen.

Figure III.8 : D.H. small intestinal biopsy-dissecting microscope appearance showing mucosa with fingers and leaves villi.

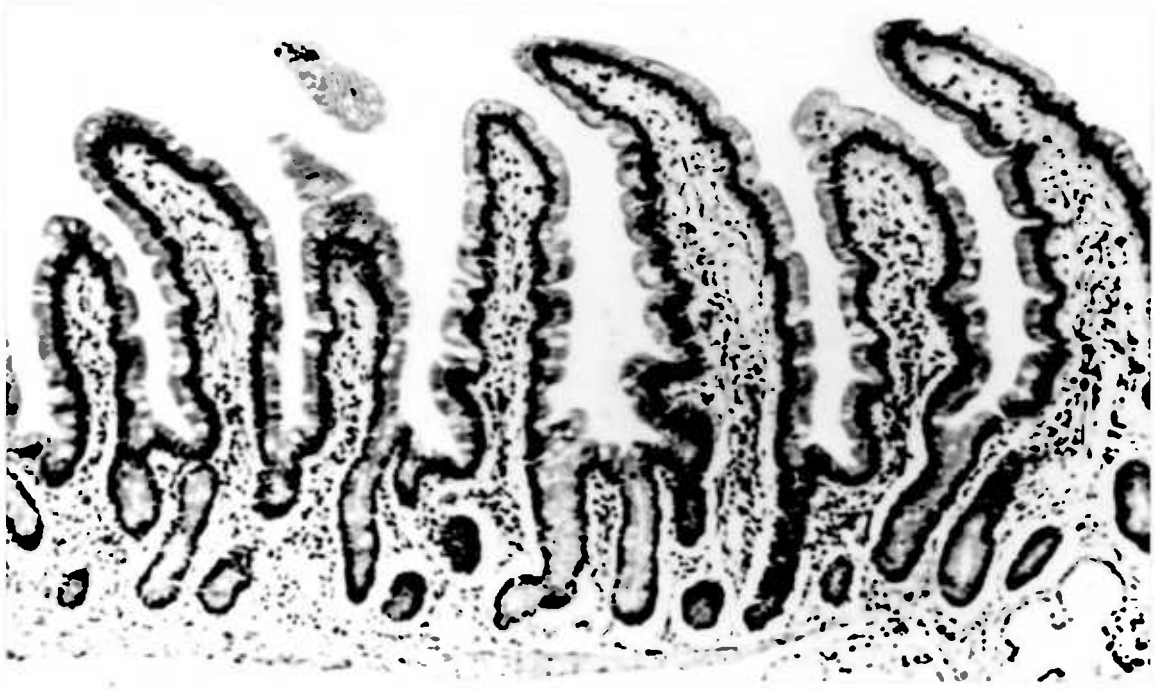


Figure III.9 : Section of mucosa shown above illustrating a typical villous configuration. Haematoxylin and eosin.

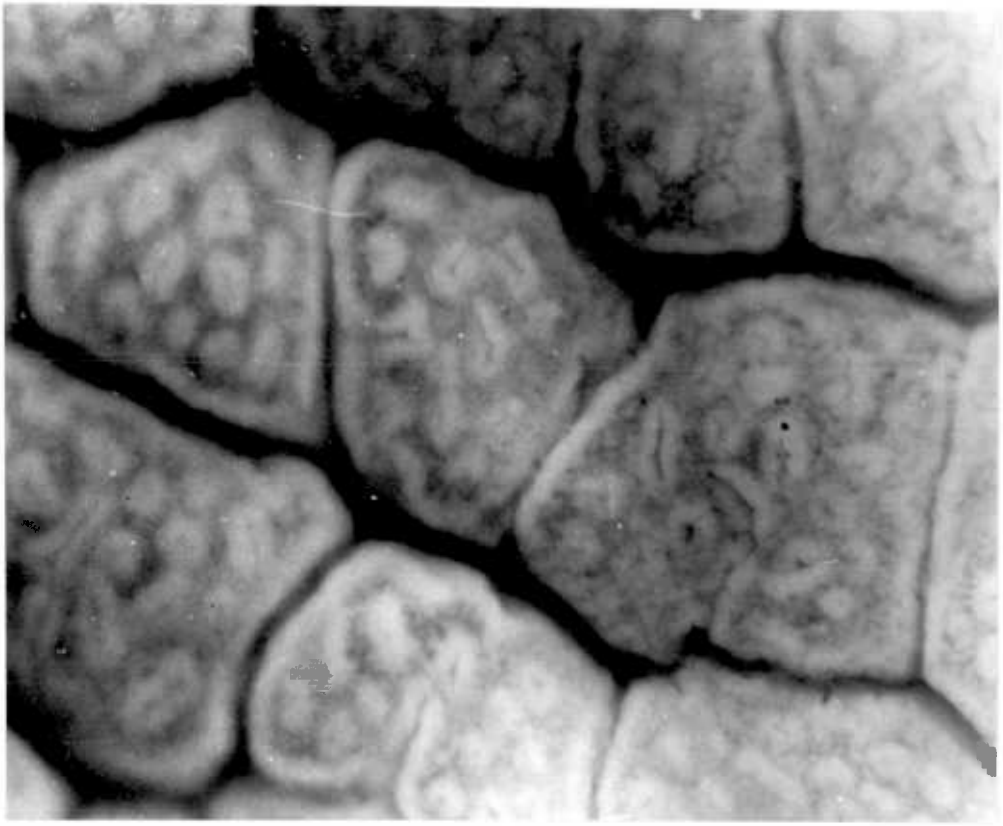


Figure III.10 : Dissecting microscope appearance showing a flat-with-mosaic mucosal pattern.

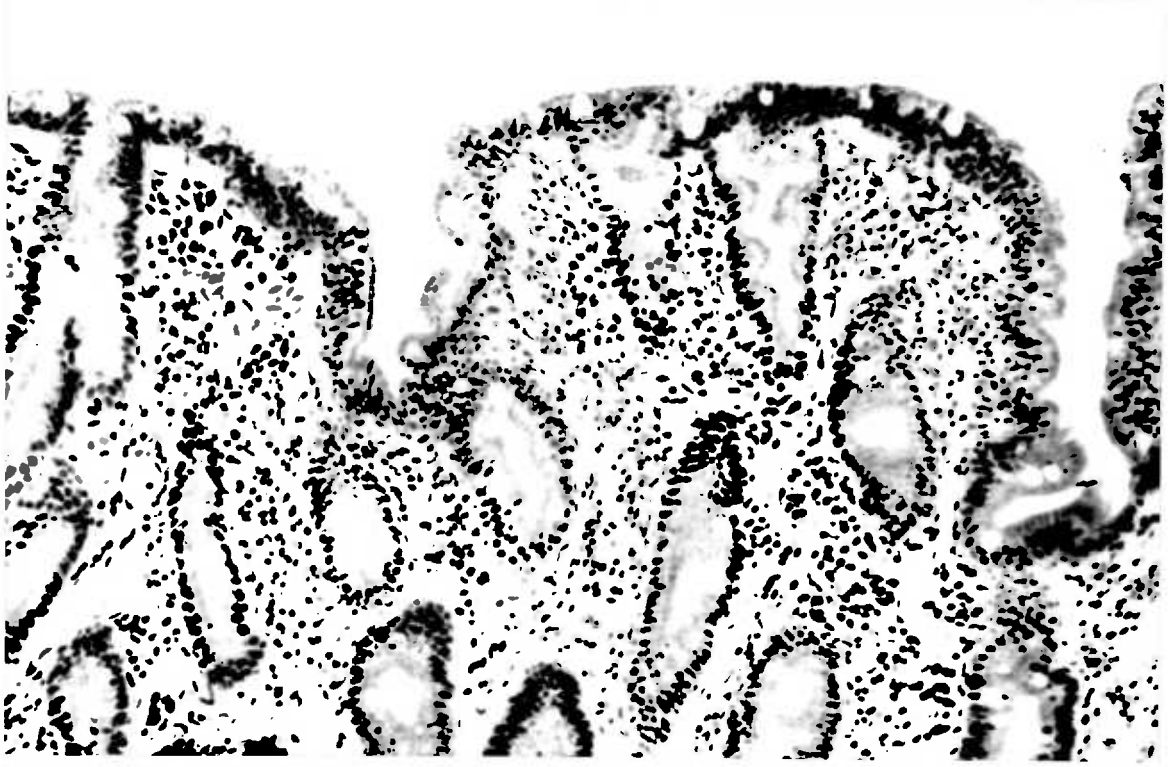


Figure III.11 : Section of the jejunal biopsy specimen shown above showing grossly abnormal pattern of the deformed villi. Haematoxylin and eosin.

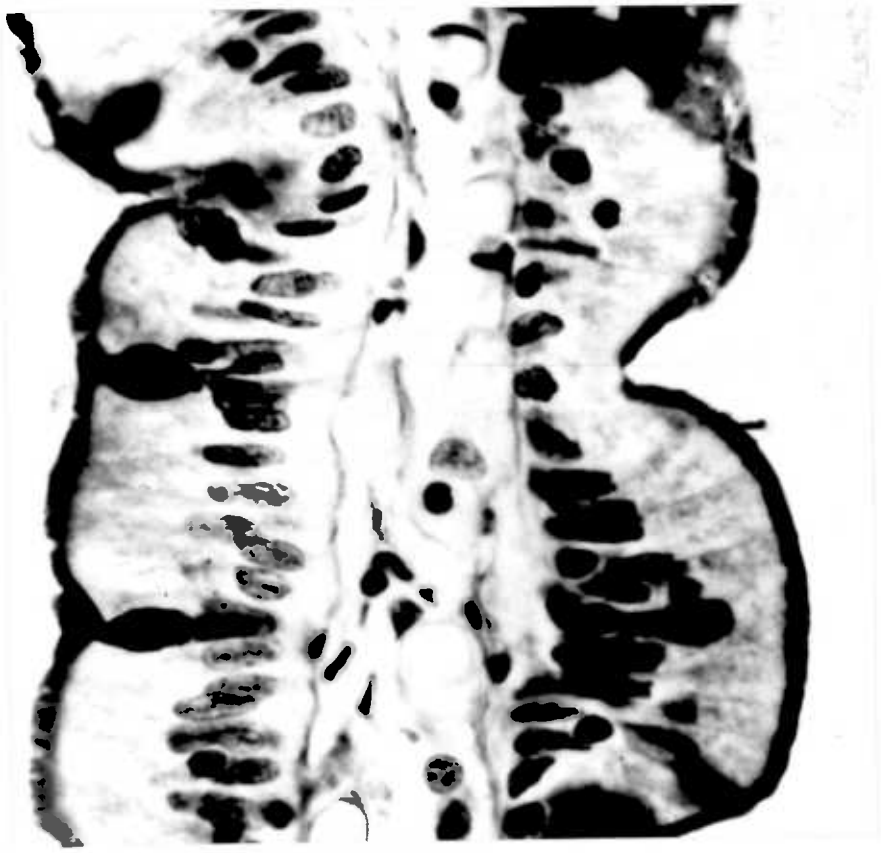


Figure III.12 : Normal villus showing columnar cells, goblet cells and minimal lymphocytic infiltration.PAS and haematoxylin.

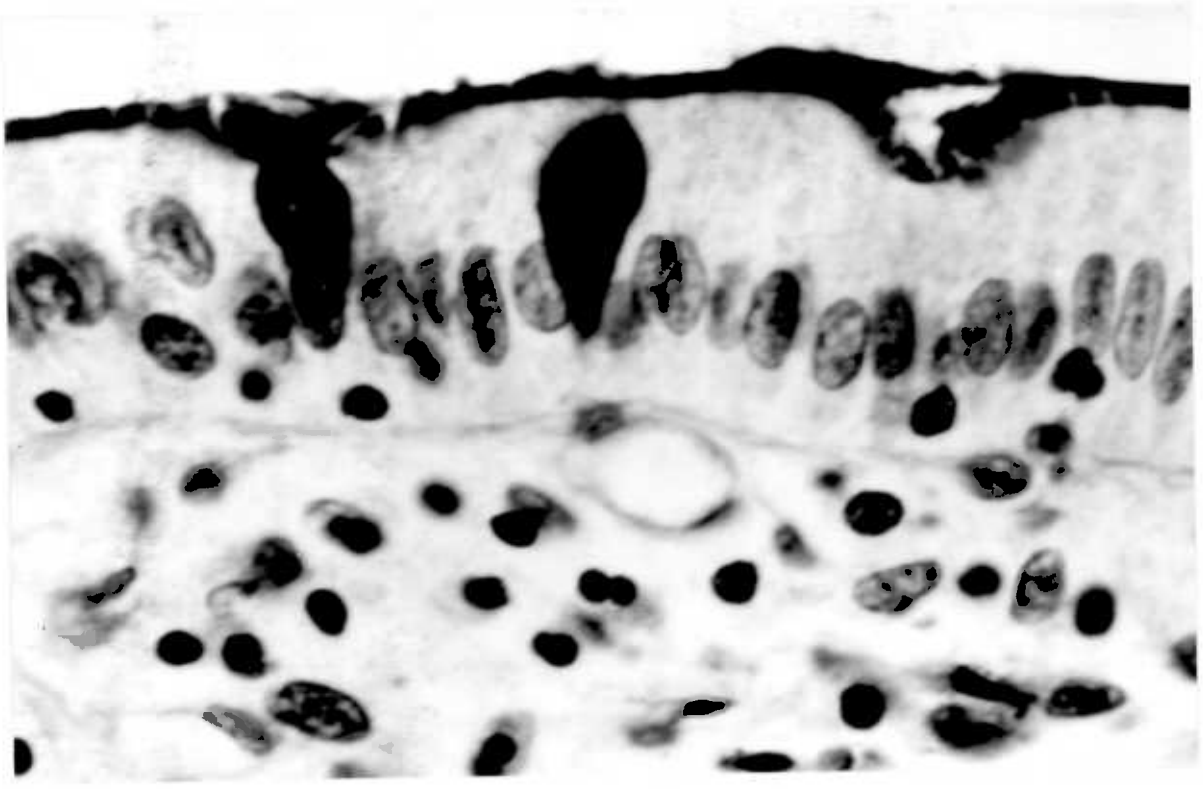


Figure III.13: Villus showing a moderate degree of lymphocytic infiltration. PAS and haematoxylin.

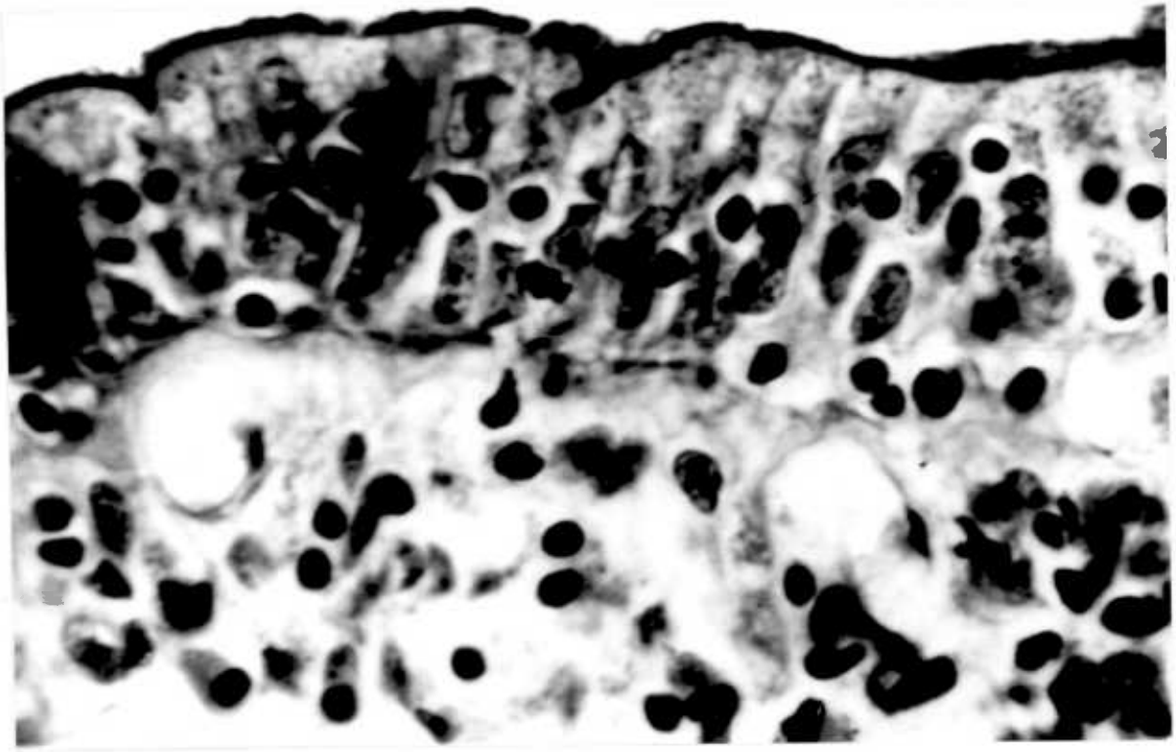


Figure III.14 : Mucosa showing heavy lymphocytic infiltration in D.H. small bowel biopsy. PAS and haematoxylin.

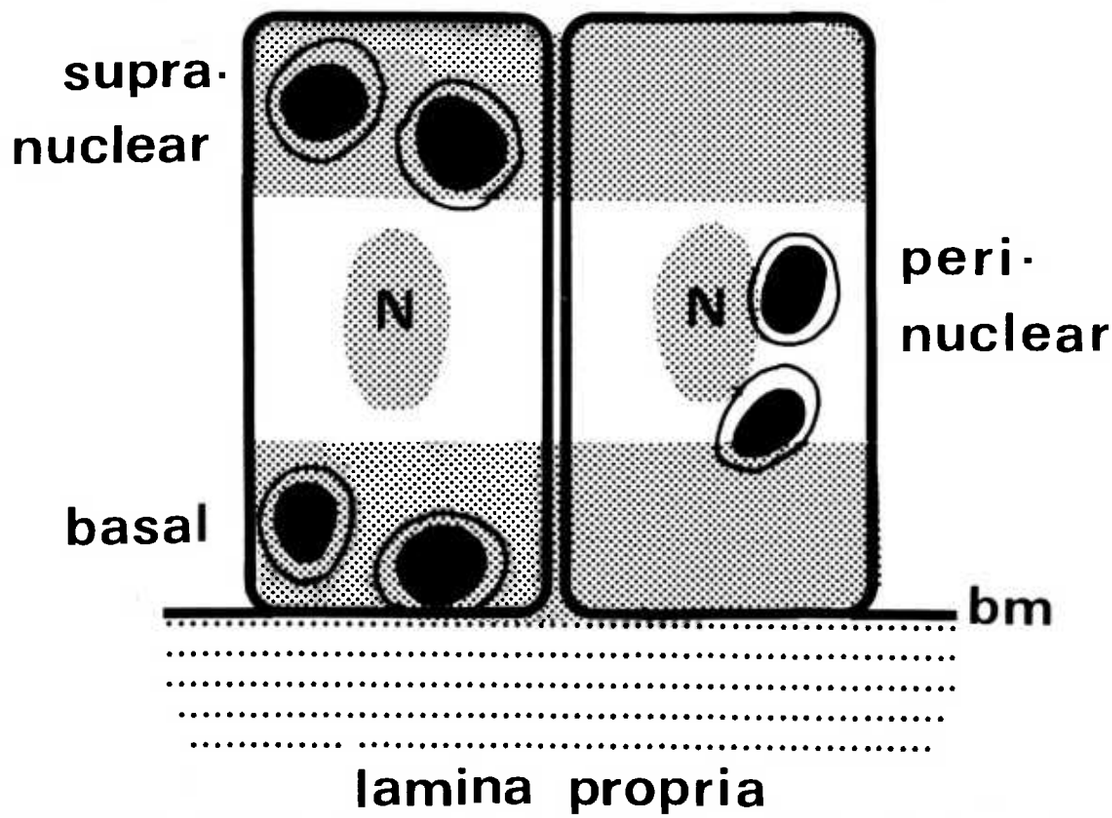


Figure III. 15 : Figure illustrating positions of intraepithelial lymphocytes in the small intestinal mucosa. Lymphocytes are allocated into 'basal', 'perinuclear' and 'Supranuclear' categories.
N = epithelial cell nucleus.

PART IV

RESULTS

Autoantibodies in Patients with Dermatitis Herpetiformis and Adult Coeliac Disease.

In this study, sera from 29 D.H. patients, 31 patients with adult coeliac disease and 28 healthy control subjects were examined. The control group consisted of 13 males (ages 20 - 79, average 47 years) and 15 females (ages 19 - 53, average 33 years).

The results are tabulated in Table IV. 1.

10 (34%) of the 29 D.H. patients had ANF of titres ranging from 1:20 to 1:160. 6 were of IgM class predominantly and 4 of IgG class. In the controls, one serum was positive (IgG 1:10), and in the adult coeliac disease patients 2 were positive (1:20 and 1:40). The increase observed in D.H. is significant ($p < 0.001$).

Gastric parietal cell antibody was found in 3 of the D.H. patients, in 4 of the adult coeliac disease patients, but not in the controls. Mitochondrial antibodies were found in one of the D.H. and 2 of the adult coeliac disease patients, but not in the controls. Thyroid microsomal antibodies were found in 5 D.H. and 3 adult coeliac disease patients, and in one control. Thyroglobulin antibody was only found in one patient (with adult coeliac disease) at a titre of 1:50.

Smooth Muscle Antibody.

A separate study was performed for the detection of smooth muscle antibody using the scoring system of Whitehouse and Holborow (1971).

Sera from the following patient groups were examined:-

Dermatitis Herpetiformis	42
Adult Coeliac Disease	42
Childhood Coeliac Disease	24
Normal adult controls	14
Children with various gastro-intestinal disorders - not coeliac disease	31
Vitiligo	10
Pernicious anaemia	5

Group	No. Tested	A.N.F.	G.P.C.	Mito.	Thyroid Microsome	Thyroglobulin
Dermatitis Herpetiformis	29	10 (34.5%)	3	1	5	0
Adult Coeliac Disease	31	2 (6%)	4	2	3	1 (titre 1:50)
Controls	28	1 (4%)	0	0	1	0

G.P.C. = gastric parietal cell antibody
Mito. = mitochondrial antibody

Table IV. 1 : Autoantibodies in Patients with D.H. and with Adult Coeliac Disease, and in Normal Healthy Controls.

Patients with various skin disorders	27
Light sensitivity	6
Urticaria	1
Penicillin sensitivity	2
Erythema nodosum	2
Pyoderma gangrenosum	1
Dapsone responsive rashes	3
Erythema multiforme	1
Herpes zoster	1
Anal condylomata	1
S.L.E.	1
Vasculitis	1
Psoriasis	4
Undiagnosed rashes	3

The results are tabulated on Table IV. 2.

Patterns of Immunofluorescent Staining obtained with ARA.

A wide variety of connective tissue staining patterns was seen with sera of patients with various diseases. More specifically, with D.H. and coeliac disease patients, more distinct patterns of staining were seen and two main types appear to predominate using rat composite block tissues:

(i) Staining of well defined adventitial fibres around blood vessels, of finer but also well defined fibres in the liver parenchyma (Figure IV.1 Similar well defined fibres were also seen in between the gastric glands of the stomach and in the submucosa. In the kidney, there was staining of well defined fibres around the renal tubules, around blood vessels, and around the glomerulus (Figure IV.3), though staining of the glomerular tuft itself was not present.

This was the main pattern of immunofluorescent staining seen, especially with the childhood coeliac disease patients, where it was very predominant.

(ii) More diffuse connective tissue staining is sometimes seen especially in the liver sinusoids and the adventitia of blood vessels. In the kidney, staining is seen around the tubules and glomerulus, sometimes

Table IV.2 : Smooth Muscle Antibody in Patients with D.H., Childhood and Adult Coeliac Disease and Various other Disorders.

Disease Group	No. Tested	Smooth Muscle Antibody Score 0-5	6-9	11-15	Positive	%
D.H.	42	31	8	3	11	26
Adult Coeliac Disease	42	31	7	4	11	26
Childhood Coeliac Disease	24	9	13	2	15	62
Normal Adult Controls	14	10	2	2	4	28
Children with G-I Disorders	31	9	18	4	27	70
Vitiligo	10	6	3	1	4	40
Pernicious Anaemia	5	3	2	0	2	20
Misc. Skin Disorders	27	14	3	10	13	48

Scoring system: 0-5 = negative
6-9 = weak positive
10-15 = strong positive

involving Bowman's capsule, and occasionally associated with weak glomerular tuft staining. It would appear that in this case, a more diffuse and finer network of connective tissue was involved (Figure IV.4).

With both these patterns, uptake and staining by all three tissues making up the rat composite block was usually seen.

Early in the study, all forms of connective tissue staining seen were recorded, but as more specimens were examined it became obvious that the pattern of staining seen with D.H. and coeliac sera were in the main, those described in (i) and (ii) above. These patterns will therefore be called 'typical' anti-reticulin antibody staining, whilst others which do not correspond will be termed 'atypical' connective tissue patterns. It should be stressed however, that this division is a purely arbitary one based on visual interpretation and impression from examining a large number of sera from various diseases. The exact nature of these antibodies and the antigens they react with will need further, more detailed investigations to elucidate their identity and significance.

Other forms of connective tissue staining seen, mainly in non-D.H. or coeliac disease patients include:-

(i) very diffuse staining, lacking definition, of a homogeneous substance suggesting uptake by ground-substance matrix rather than connective tissue.

(ii) isolated staining of liver sinusoids alone (Figure IV.5) not associated with adventitial staining, or uptake by stomach and kidney tissues. Occasionally, Kupfer cell staining is also seen.

(iii) isolated staining of glomerular tuft and blood vessels with absence of peritubular, periglomerular uptake, (Figure IV.6) and negative staining of the liver and stomach.

(iv) isolated staining of the intergastric connective tissue in a linear fashion, with absent uptake by the submucosa, kidney and liver.

(v) isolated staining of the adventitial tissue round blood vessels in a linear fashion with absence of peritubular, periglomerular, intergastric and hepatic uptake (Figure IV. 8).

(vi) isolated staining of connective tissues around the portal veins with absent staining elsewhere (Figure IV. 9).

The number and permutations of these patterns are numerous and the foregoing only describe some of them. The numbers involved are small and not particularly confined to any one particular disease group. They also crop up with equal frequency in D.H. and coeliac disease patients and very occasionally they are seen in the serum of apparently healthy normal controls.

Correlation of ARA Fluorescent Staining Patterns with Histological Stains for Connective Tissue Components.

When compared with histological stains for reticulin (Gomori silver stain) and for elastin and collagen (van Giesen), the best correlation obtained with the fluorescent pattern was that of the histological stain for reticulin (compare Figure IV. 1 with Figure IV. 7). This applied to all three tissues examined with the rat composite block. No correlation with elastin or collagen was evident.

For these reasons, the term 'anti-reticulin antibody' was used to describe the connective tissue antibodies found in the patients with D.H. or coeliac disease. The implication here is that these antibodies probably react with an antigen involved with, or located in, the reticulin fibres detected by silver staining. The comparison therefore, is a purely histochemical one; it is not a biochemical definition and it does not necessarily implicate biochemical identity.

With the 'atypical' connective tissue antibodies described above, no consistent correlation with any one particular staining for connective tissue was apparent, suggesting a marked heterogeniety of antigens involved.

Reaction of ARA with Human Tissue.

When tested against human foetal (16 weeks) liver (Figure IV. 2) and small intestine (Figure IV. 11) cryostat sections positive staining was seen

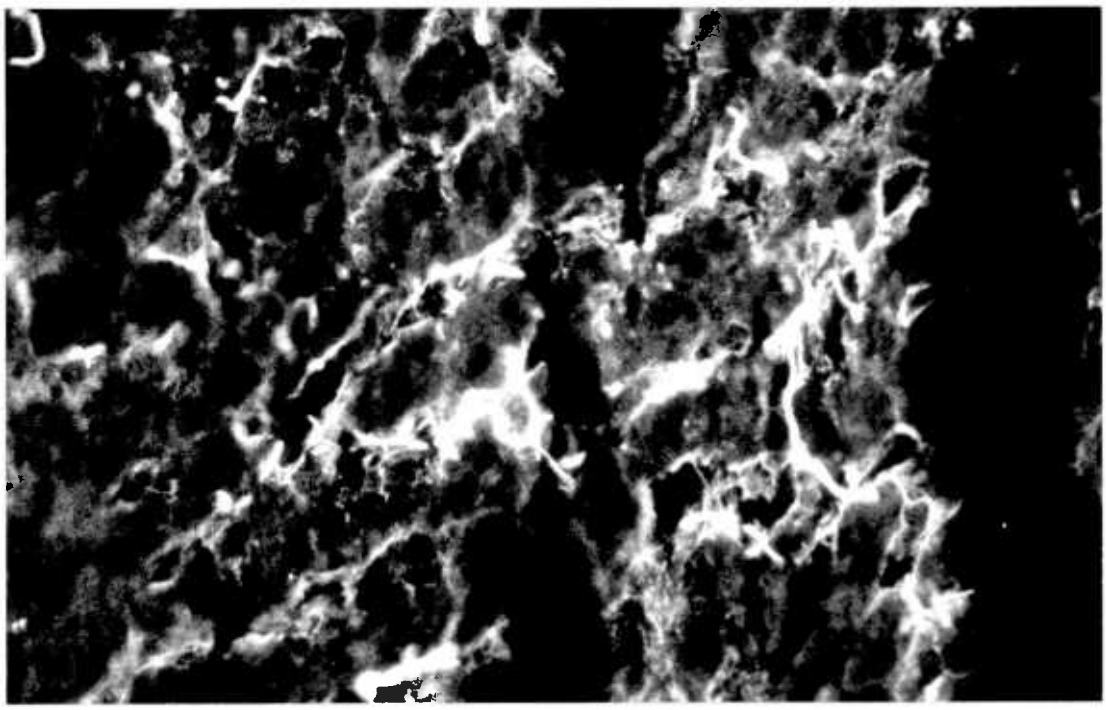


Figure IV. 1 : Anti-reticulin antibody - IgG class from patient with adult coeliac disease showing well defined staining of reticulin fibres in the parenchyma of rat liver.

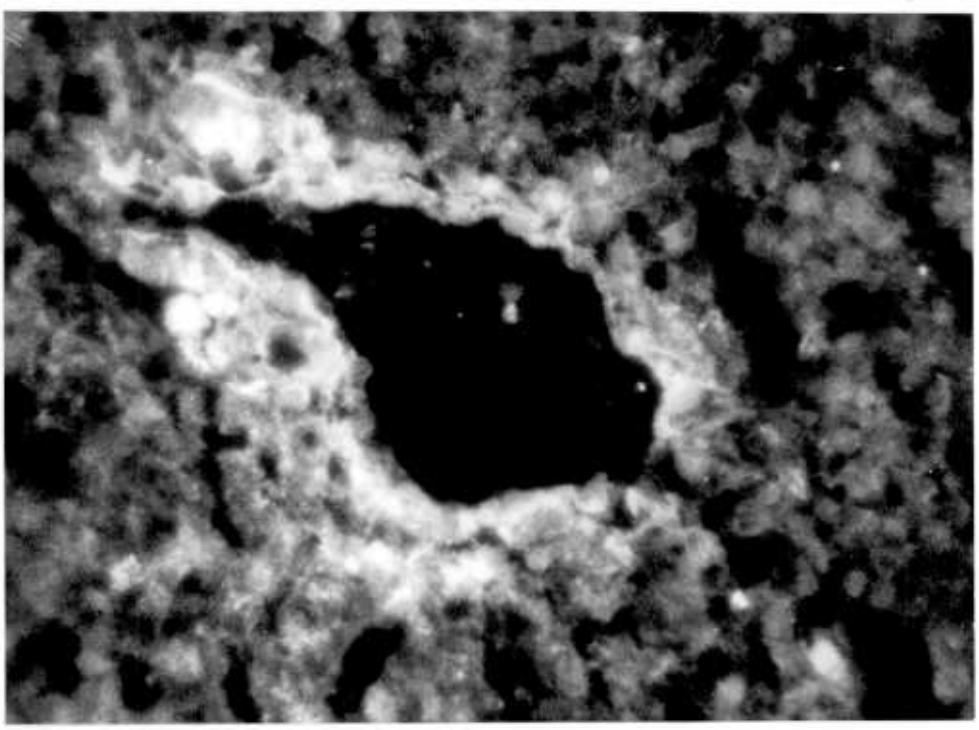


Figure IV. 2 : Anti-reticulin antibody - IgG class from D.H. patient showing staining of connective tissue in portal tract of human foetal (16 weeks) liver.

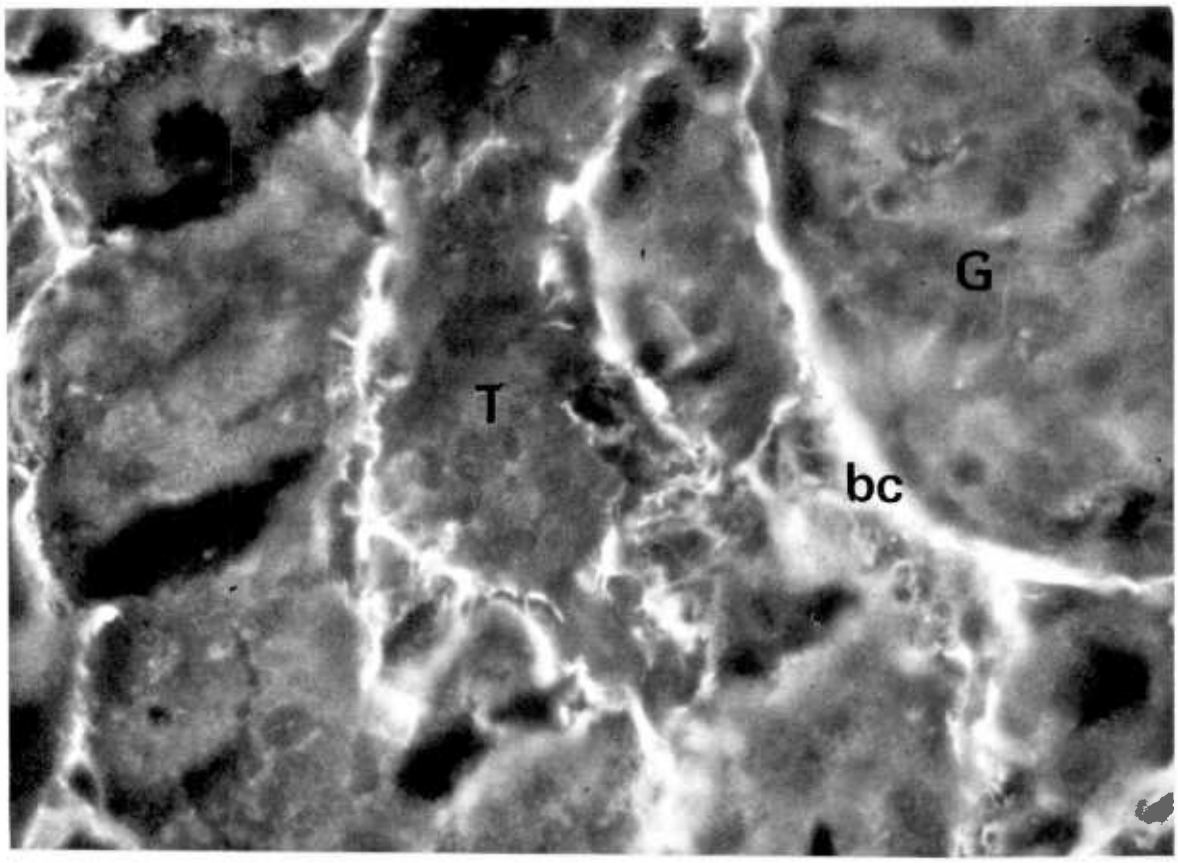


Figure IV. 3 : Anti-reticulin antibody -IgG class from DH patient showing staining of peri-tubular and peri-glomerular reticulin fibres in rat kidney. G=glomerulus; T=tubules; bc=Bowman's Capsule

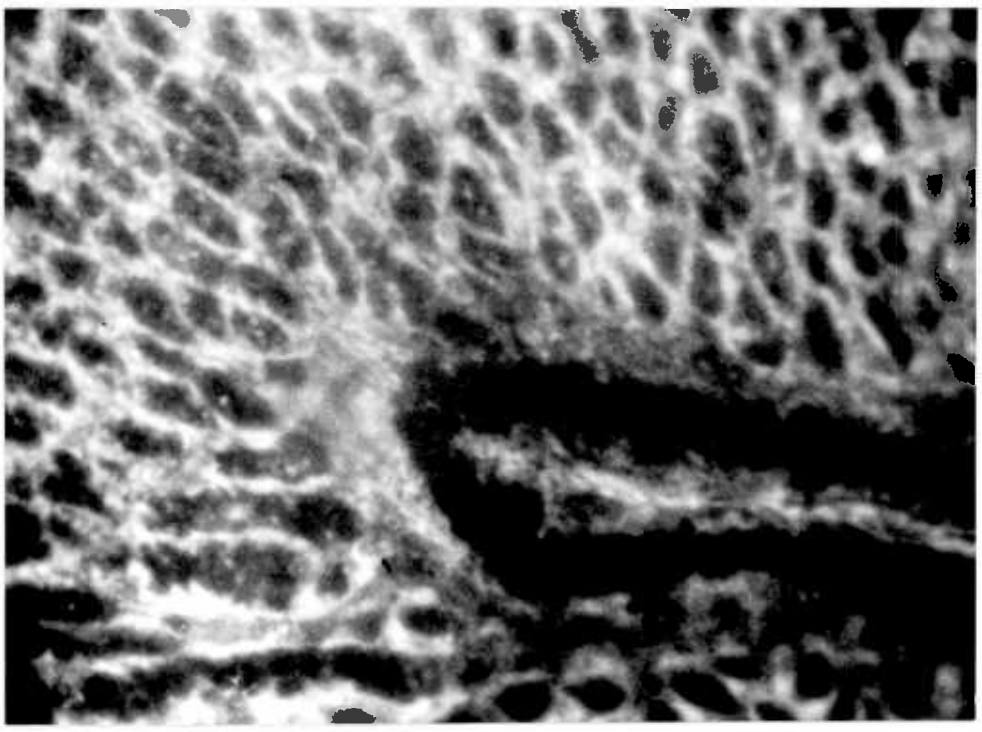


Figure IV. 4 : Anti-reticulin antibody - IgA class from patient with childhood coeliac disease showing staining of connective tissue fibres between the gastric glands and in the submucosa of rat stomach.

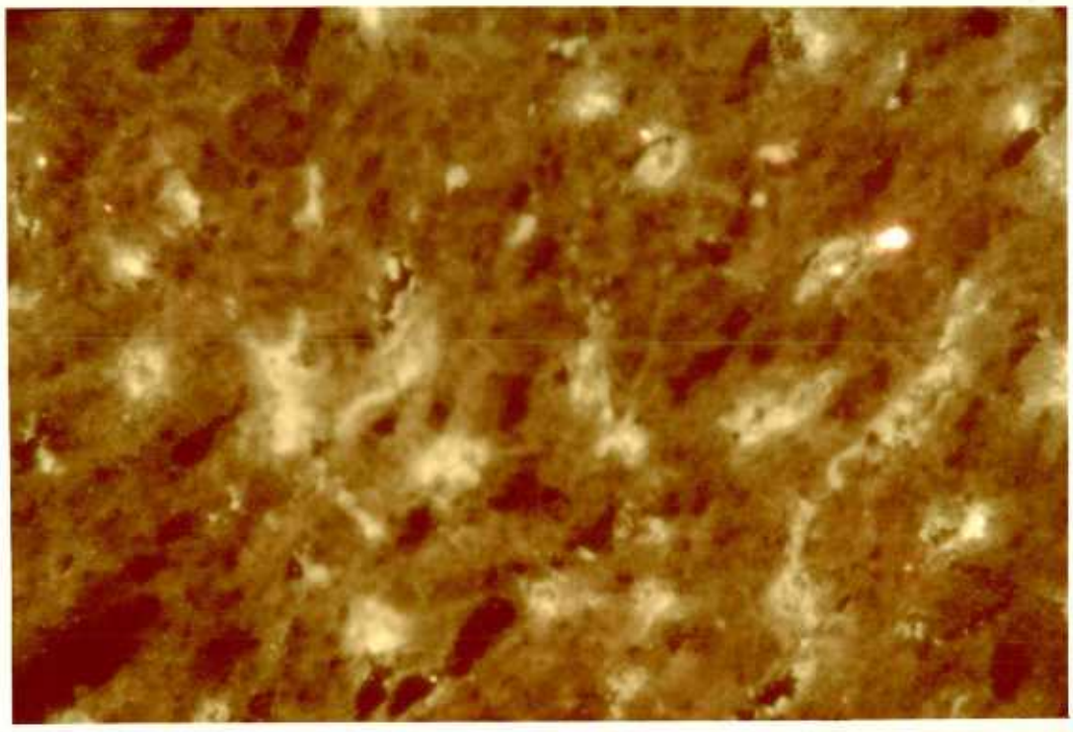


Figure IV. 5 : Atypical connective tissue staining seen in the serum of a non-DH or coeliac patient showing diffuse involvement of the liver sinusoids - unlike the well defined pattern seen in figureIV. 1 .

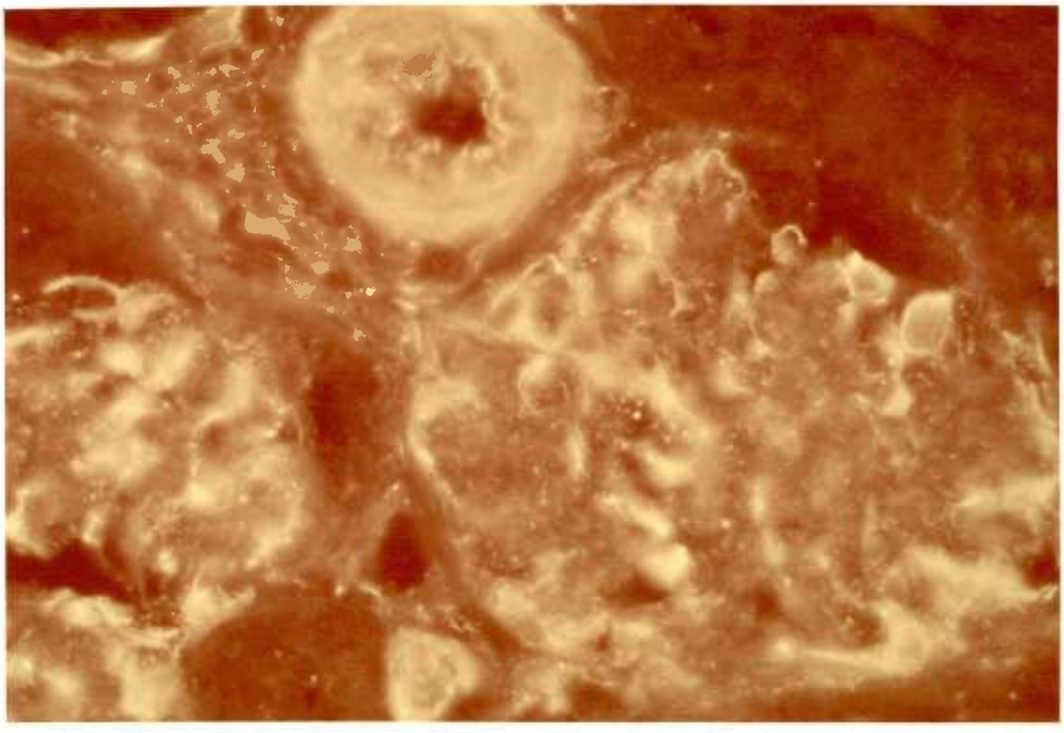


Figure IV. 6 : Atypical connective tissue pattern seen in a non-DH or coeliac patient showing well defined staining in the renal glomerulus and blood vessel wall of rat kidney. Glomerular staining of this magnitude is not seen with anti-reticulin antibody.

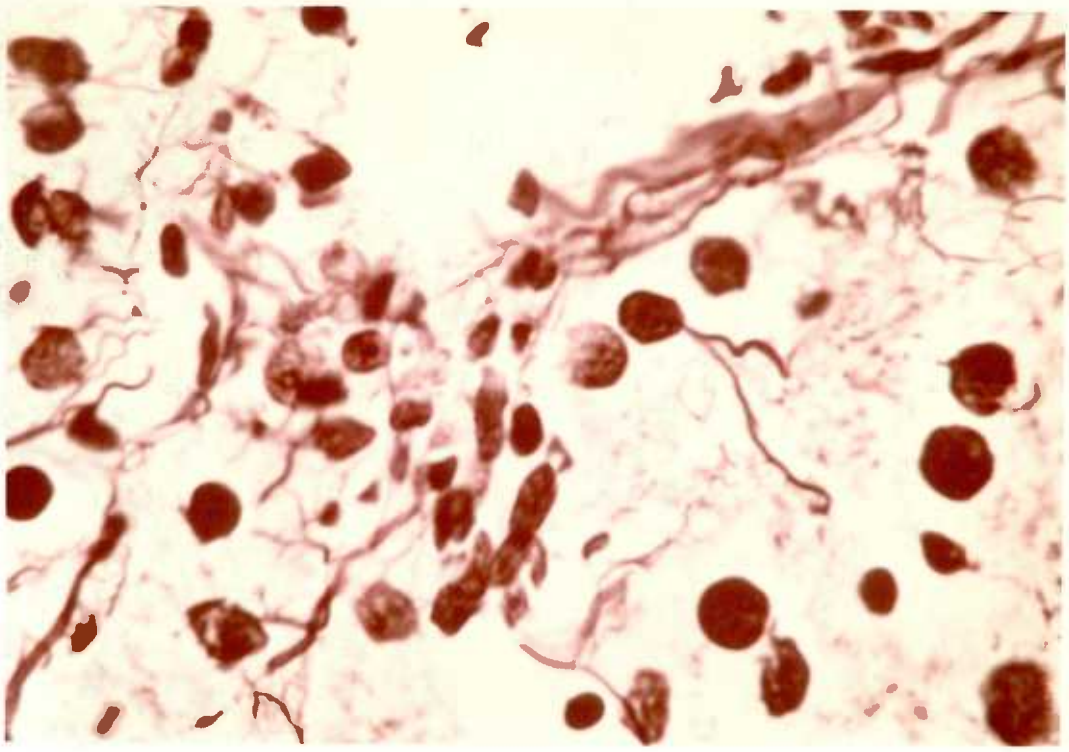


Figure IV. 7 : Rat liver - Gomori silver stain for reticulin showing well defined connective tissue fibres similar to those shown by immunofluorescence in figure.IV.1

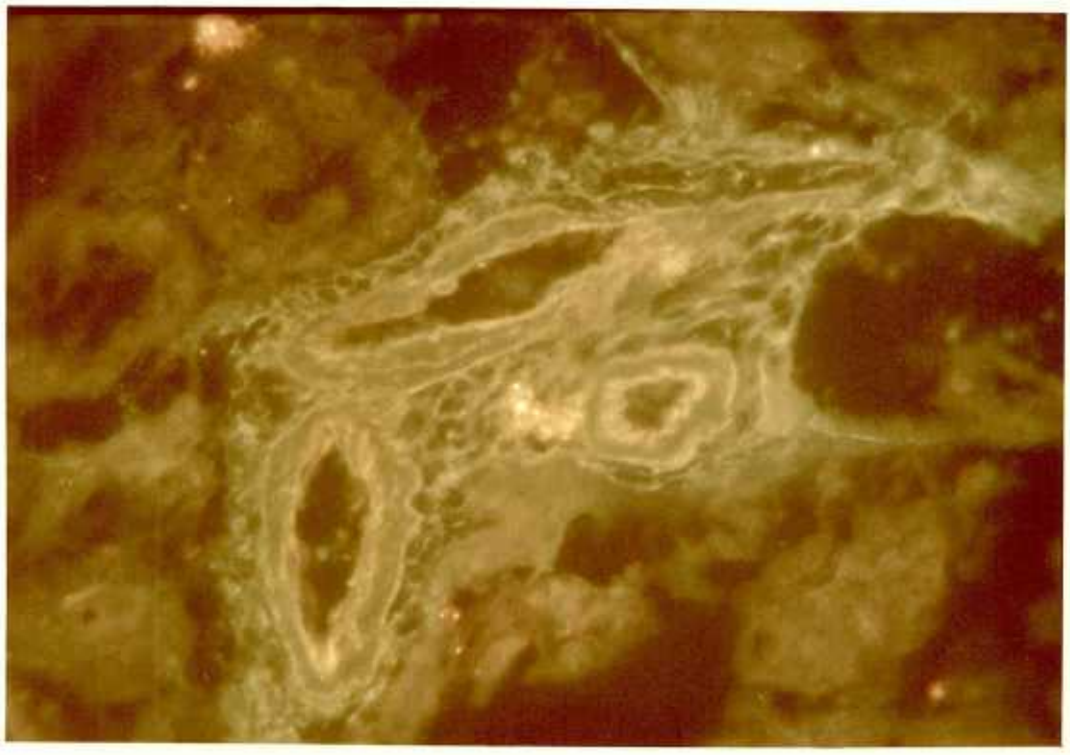
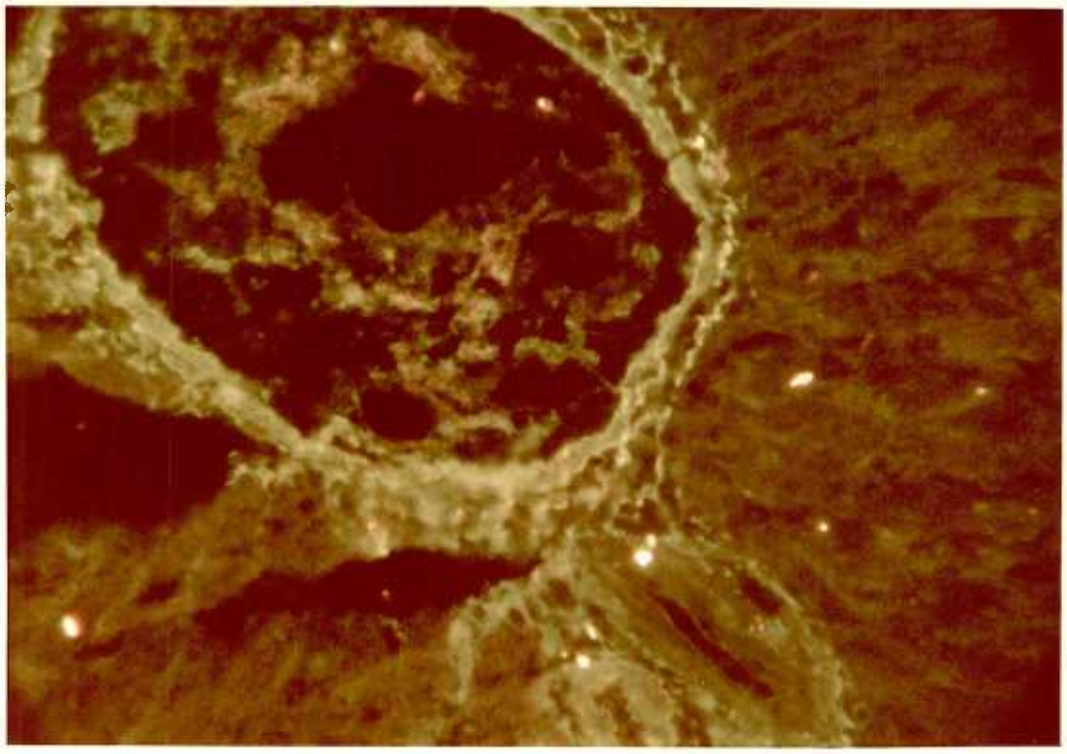


Figure IV. 8 : Atypical connective tissue staining: detected by indirect immunofluorescence in serum of patient with pemphigus. Rat kidney, showing staining of connective tissue around blood vessels. No staining is seen in the peri-tubular region of the renal tubules.



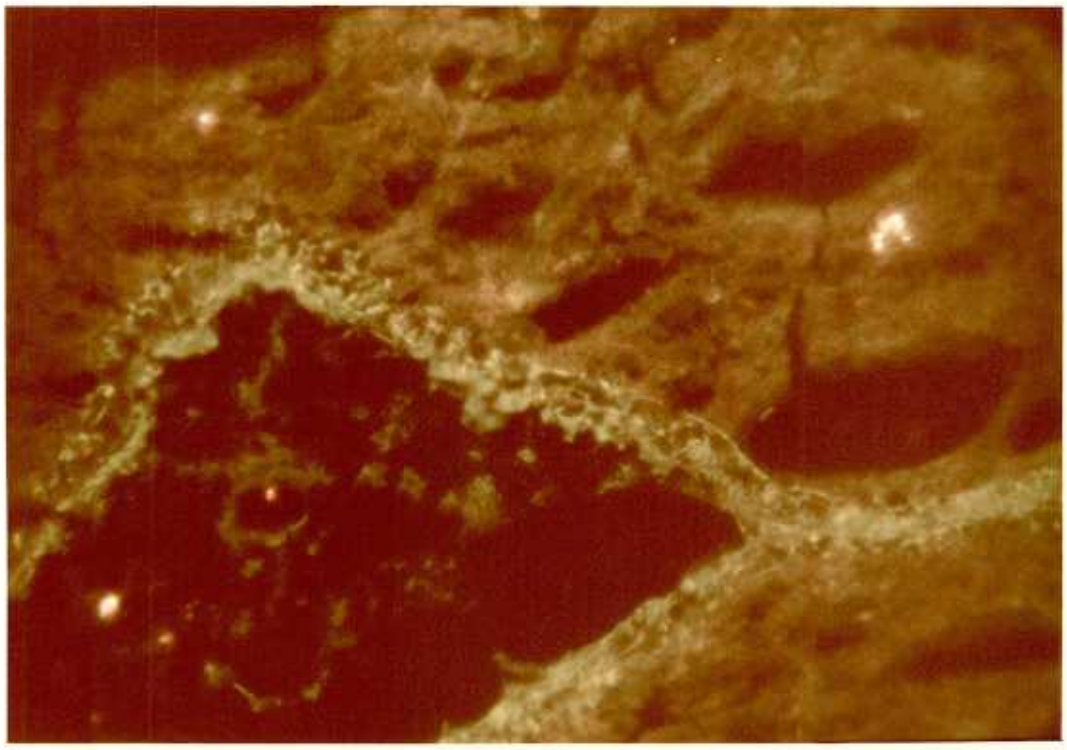
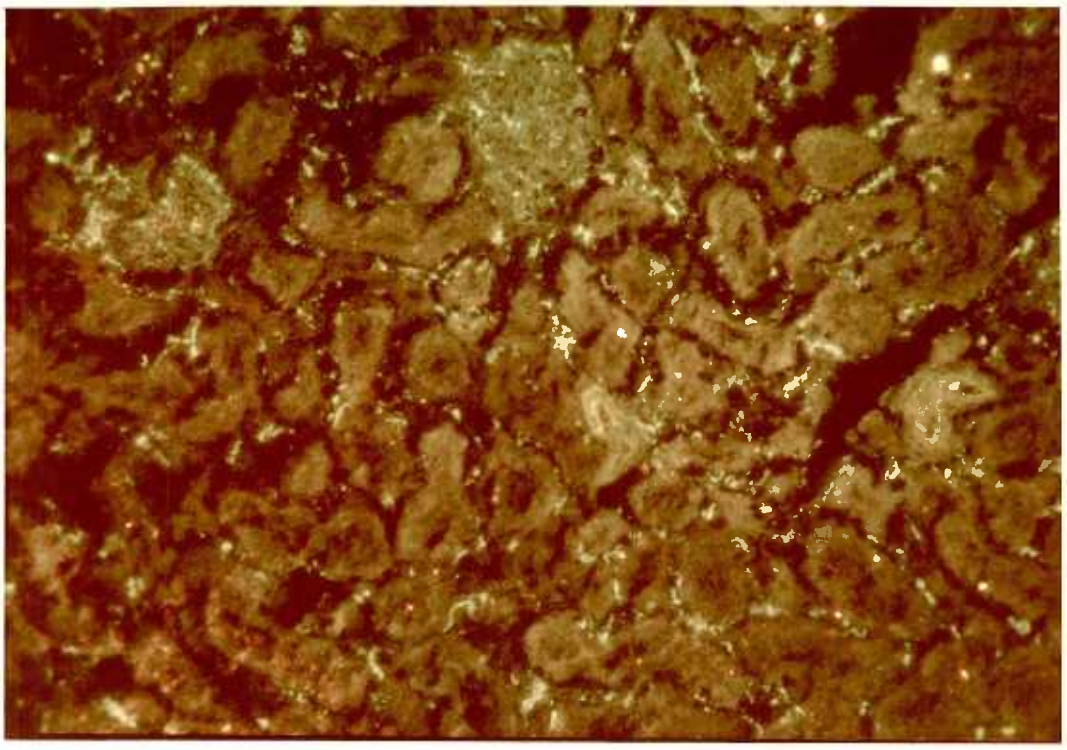


Figure IV. 9 : Atypical connective tissue staining patterns showing well defined staining of fibres around the cetrilobular vein in rat liver. Detected by indirect immunofluorescence in the serum of a patient with chronic active hepatitis. No staining is seen within the liver parenchyma itself.



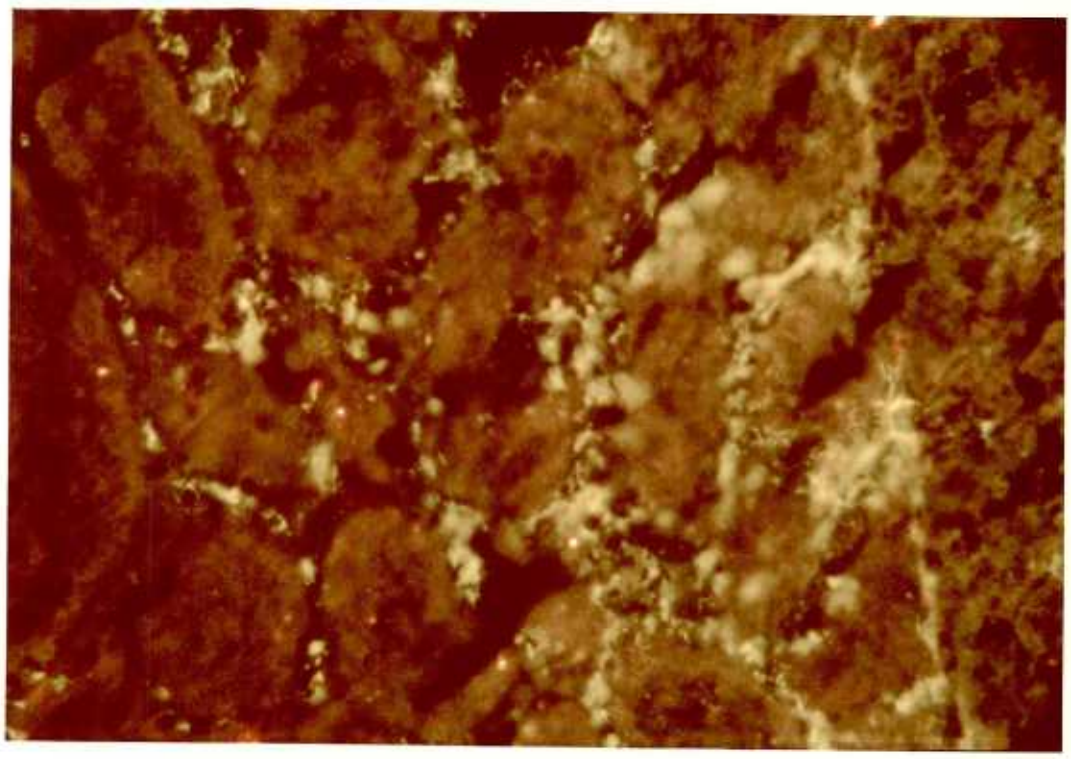


Figure IV.10 : Atypical connective tissue pattern detected in the serum of a non-DH orcoeliac patient. Low and high power views showing staining of peri-tubular and peri/intra-glomerular tissue but in a pattern quite unlike that seen in figure IV. 3 .

with ARA positive sera (as detected by using rat tissue). In the liver, staining of the adventitial tissue in a portal tract was seen. In the small intestine, a diffuse network of reticular fibres was seen, involving and confined to the lamina propria (Figure IV. 11).

The ability of these antibodies to react with human tissues suggest that these antibodies are directed against autoantigens. In this respect therefore, anti-reticulin antibody detected in these patients can be called and 'autoantibody'.

With normal adult skin however, staining with anti-reticulin antibody was consistently negative, though staining was seen using foetal (16 weeks gestation) skin. The possible significance of this will be discussed later.

Effect of Collagenase on ARA Staining and Histological Staining.

Pretreatment of cryostat sections of rat composite blocks with two concentrations of collagenase (0.5 mg per ml and 1 mg per ml) failed to remove the ability of the connective tissue to stain with ARA positive serum by immunofluorescence. The slides incubated for 30 minutes to 3 hours showed very little difference in the intensity of immunofluorescent staining when compared with the normal control and buffer-only incubated control sections. In the latter sections, incubation with buffer-only did not appear to affect the intensity of staining.

In the 18 hour treated section the intensity of immunofluorescent staining was actually increased following collagenase treatment. Such a change was not observed with the 'buffer-only' control.

The efficacy of collagenase treatment was confirmed by the van Giesen stains done on sections treated in parallel. Van Giesen staining was abolished fairly early and had disappeared completely in the 1 hour incubated sections (see Figure IV. 12 and IV. 13). Buffer treated control sections retained their van Giesen reactivity throughout the 18 hour incubation.

With Gomori - staining for reticulin - no evidence of removal of

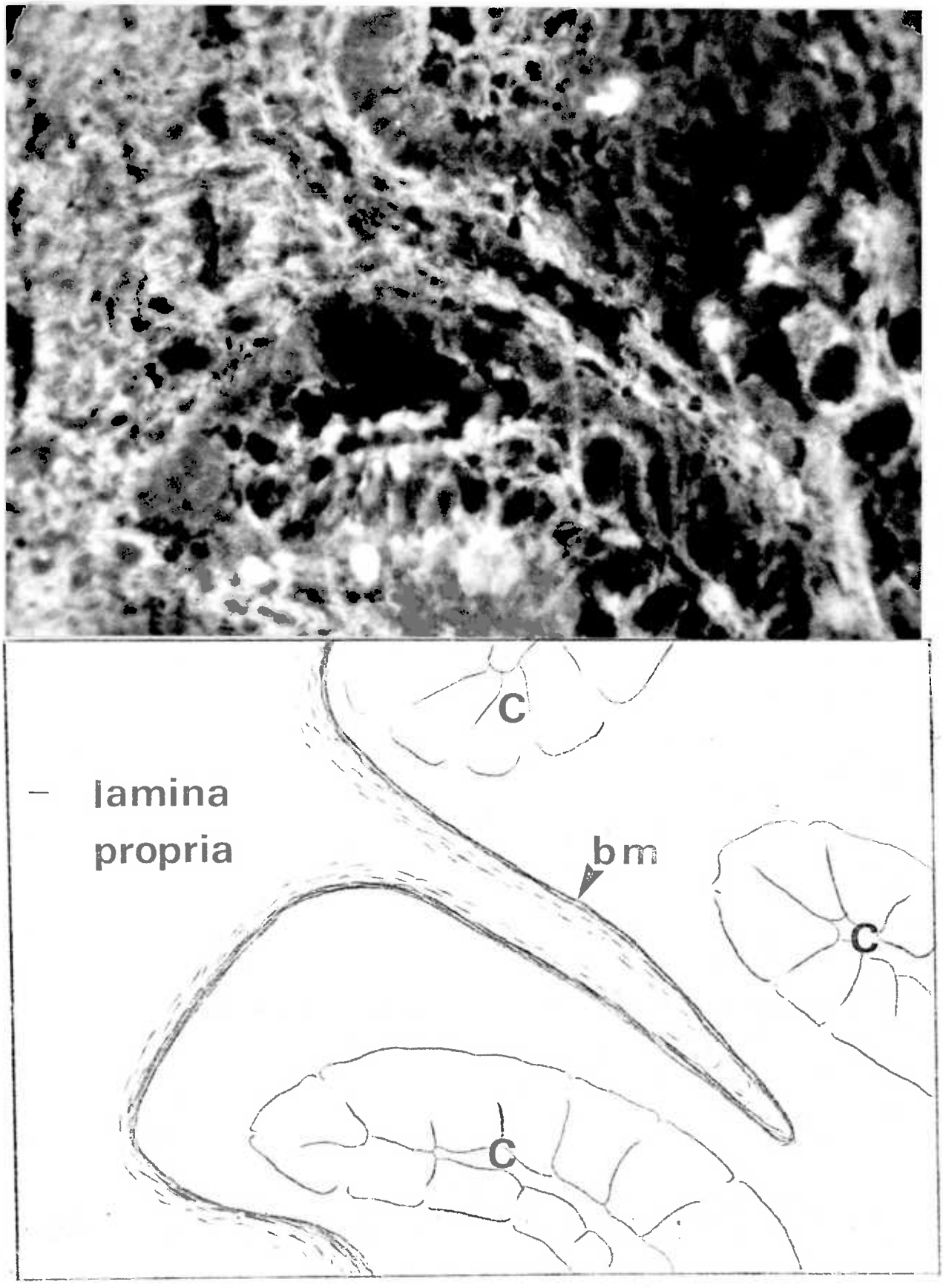


Figure IV. 11 : Human foetal jejunum (16 weeks gestation) stained with anti-reticulin antibody of IgG class from a D.H. patient and FITC sheep-anti-human IgG. Fine fluorescence of the reticular network in the lamina propria is seen, mainly in the region immediately below the basement membrane (BM). C = crypts of small intestinal epithelium.

reactivity with silver stains was seen with either the collagenase incubated or buffer-only incubated sections, suggesting that these fibres were not destroyed by the collagenase.

No immunofluorescent staining was seen in any of the test or control slides when tested with the negative ARA (normal) control serum.

Conclusions to be drawn from these studies.

These results indicate that the antigens that react with ARA positive sera are resistant to collagenase treatment. The complete removal of van Giesen reactive material and the retention of Gomori silver stain reactive fibres further supports the concept that the ARA is directed against antigen(s) associated with, or present in, reticulin fibres.

The increased intensity of ARA staining seen in the 18 hour section is interesting. It suggests that following collagenase treatment, certain new (or further) antigens are unmasked, and this could account for the increased intensity of immunofluorescent staining.

Immunoglobulin Class of ARA.

Using mono-specific FITC labelled anti-human IgG, IgM and IgA conjugates in appropriate dilutions, the immunoglobulin class of 14 ARA positive sera from patients with childhood coeliac disease was examined. The results are shown in Table IV. 4. The original selection of these 14 positive sera was made using a polyvalent anti-human immunoglobulin conjugate.

It can be seen that in all 14 sera tested, ARA of IgG class was present. In 9 of these 14, IgA class antibodies were also present. IgM antibodies were not detected at all.

Further studies on positive sera in further patients with both adult and childhood coeliac disease and in D.H. showed similar trends. IgG was invariably present and occasionally, IgA. IgM antibodies have never been detected.

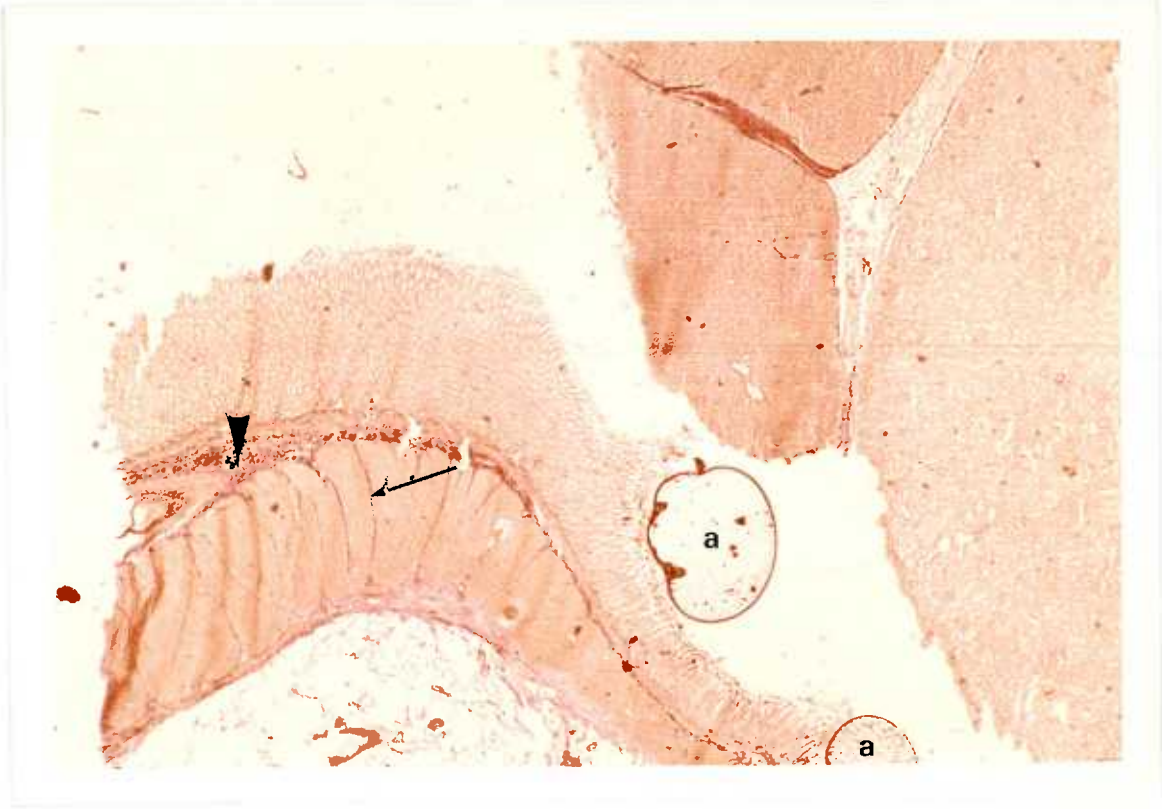


Figure IV. 12 : Rat stomach - van Giesen stain for collagen fibres (arrows).a = artefact from air bubble in mountant.

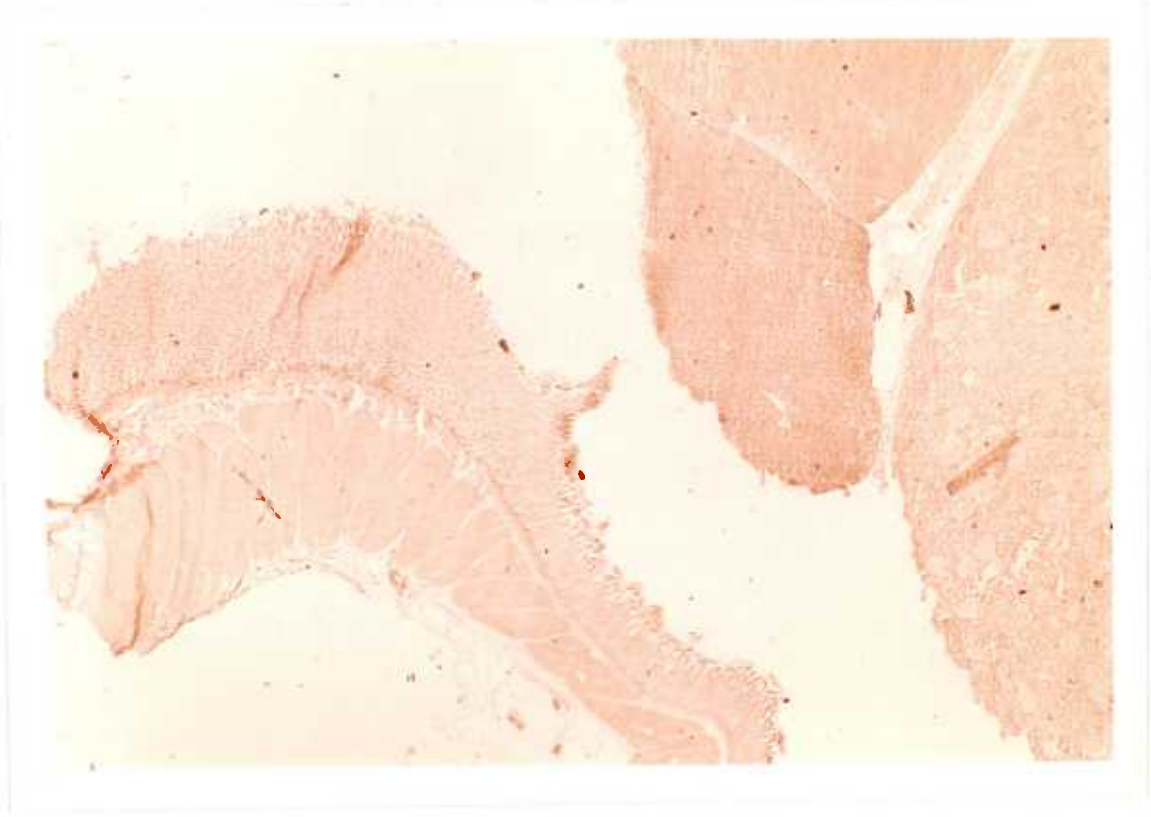


Figure IV. 13 : Same section as above after collagenase treatment showing the complete removal of van Giesen stain positive material. A similar section , when treated with anti-reticulin antibody positive serum showed an unaltered immunofluorescent pattern .

Table IV. 3 : Immunoglobulin Class of 14 Positive A.R.A. Sera from Children with Coeliac Disease.

No.	IgG	IgA	IgM
1	+	+	-
2	+	+	-
3	+	-	-
4	+	+	-
5	+	+	-
6	+	-	-
7	+	+	-
8	+	+	-
9	+	-	-
10	+	+	-
11	+	-	-
12	+	+	-
13	+	-	-
14	+	+	-
Total	14	9	0

Immunoperoxidase Staining and Immunoelectron Microscopy using ARA Positive Staining.

At light microscopic level, well defined staining of connective tissue fibres in the adventitial areas and in the liver parenchyma is seen (Figures IV. 14 to IV 17). The pattern of staining seen was identical with that obtained by immunofluorescence and by Gomori silver staining for reticulin.

Suitable thin sections in araldite (Figure IV. 17b) were subsequently processed at electron microscopic level. Under this much higher magnification, it can be seen (Figure IV. 18) that inter-hepatocyte structures were stained in well defined bundles. These bundles had a fibrillar appearance, and on higher magnification they could be identified as collagen fibres from the periodicity of the cross-banding on the fibres (Figure IV. 19) which could be calculated and were found to be in the region of 10^{3}A°.

Further examination however, revealed that the collagen fibres themselves, were not taking up the horse-radish peroxidase. Instead, they appeared relatively unstained (and this was most clearly seen in cross-sections as in the bundles of fibres shown in Figure IV. 20); the surrounding 'matrix' surrounding the collagen fibres themselves appeared to be the main structures taking up the horse-radish peroxidase.

Testing for Ability of ARA to fix Complement in vitro.

Using the method described, of the 12 ARA positive sera tested, only 1 showed complement fixing ability. This was a D.H. patient on treatment with dapsone only. IgG was the only class of ARA present in this serum. IgM and IgA antibodies were not found. With de-activated fresh human serum as diluent, no positive immunofluorescence was seen with this serum. In the gastric parietal cell control, immunofluorescence using FITC anti-C3 was also abolished when de-activated human serum was used as diluent.

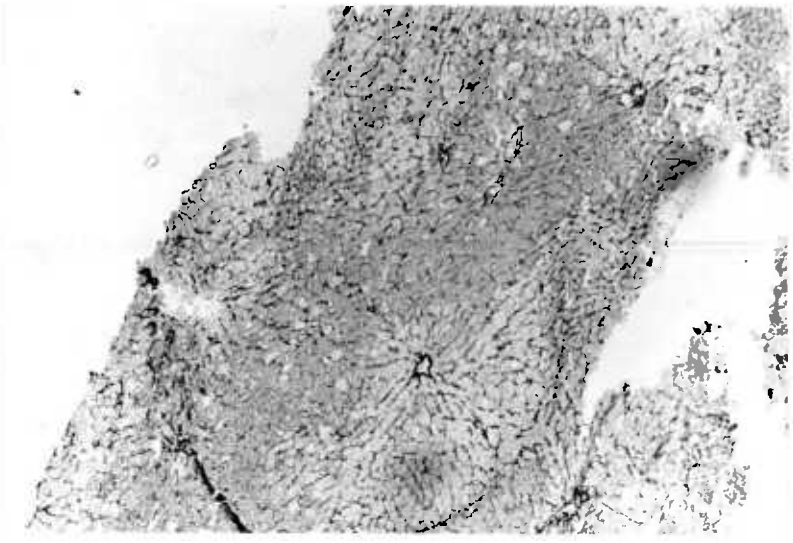


Figure IV. 14
ARA positive serum-detected using horse-radish peroxidase anti-human IgG.
Rat liver.

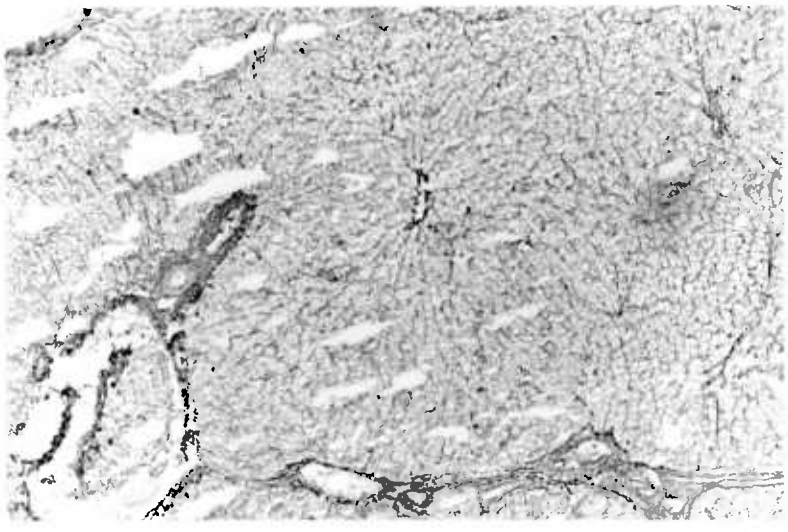


Figure IV. 15
Same section as above.

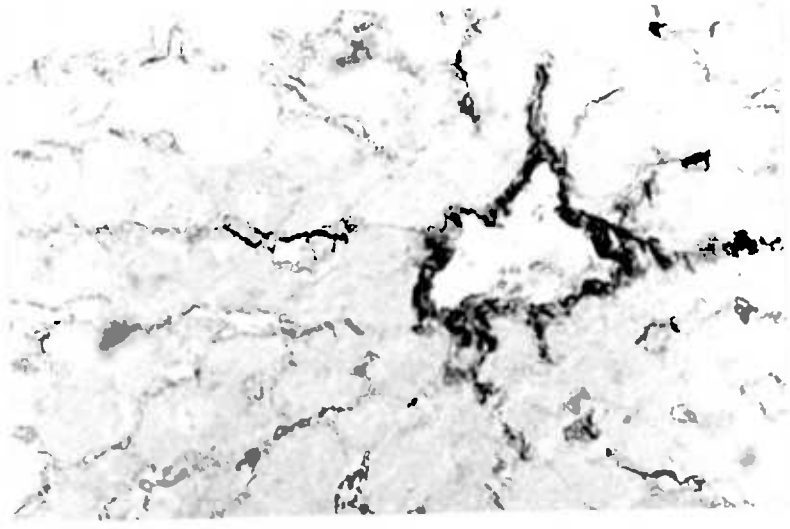
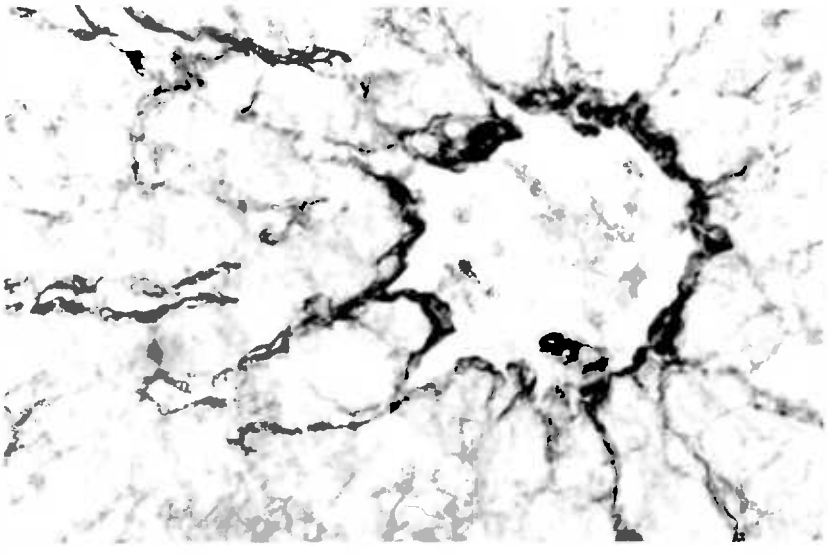
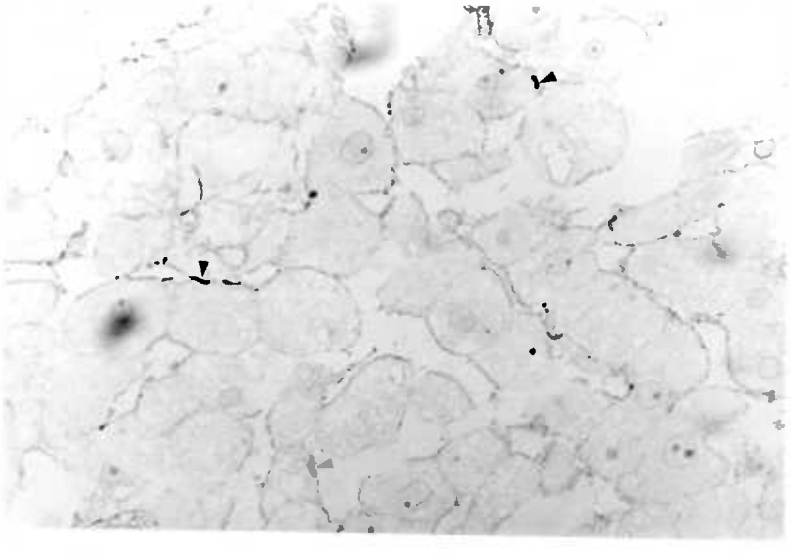


Figure IV. 16
HP view of centri-lobular vein in figure IV. above.



a



b

Figures IV.17 a and b.
ARA positive serum from patient with adult coeliac disease - detected by horse radish peroxidase conjugated anti-human IgG. Rat liver (a) HP view of centrilobular vein. Note well defined strands of connective tissue in parenchyma.

(b) 0.5 µ araldite thin section showing well defined connective tissue fibres (arrows) around hepatocytes.

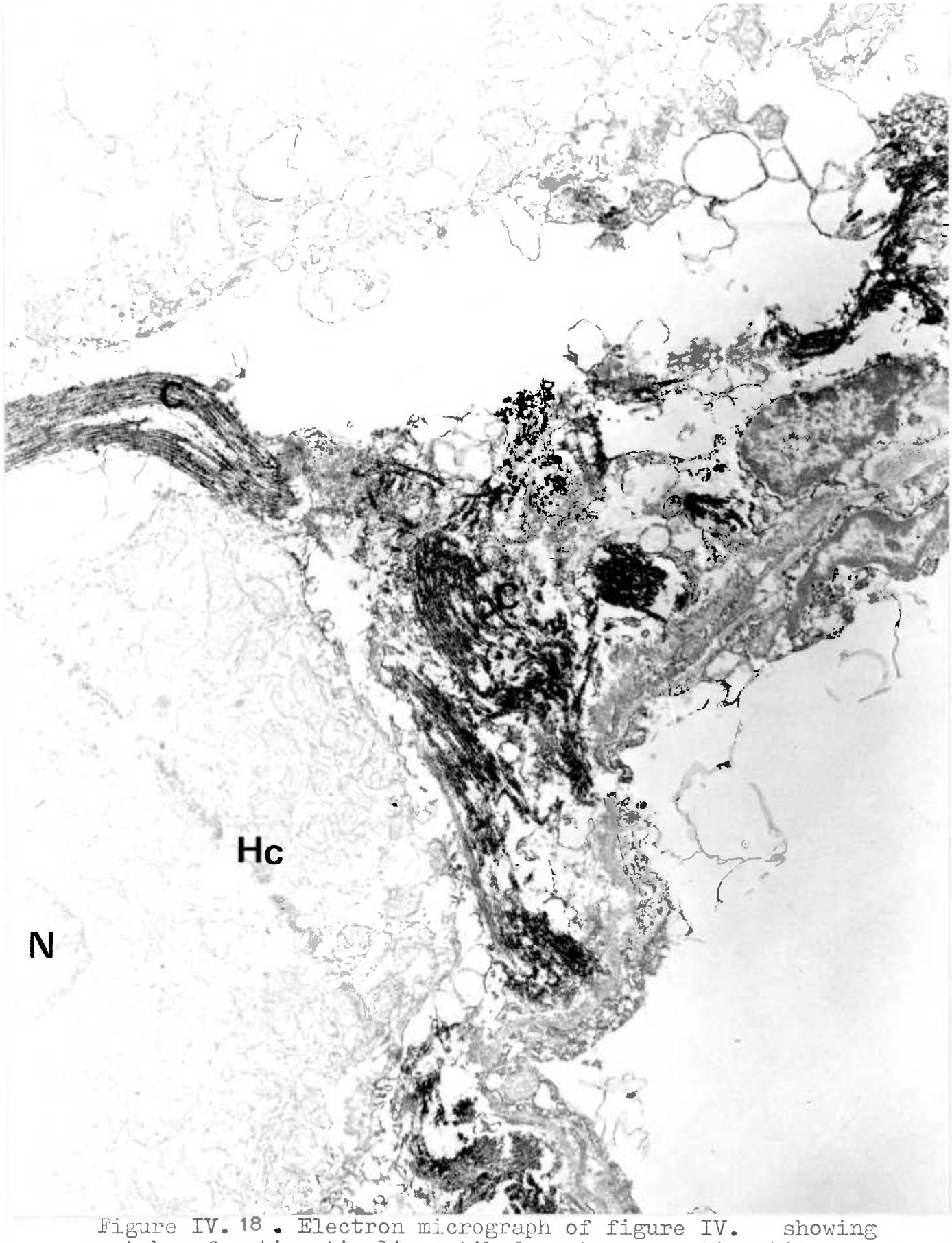


Figure IV. 18 . Electron micrograph of figure IV. showing uptake of anti-reticulin antibody onto connective tissue fibres (C) around hepatocyte (Hc).

N = hepatocyte nucleus. x 9,750.

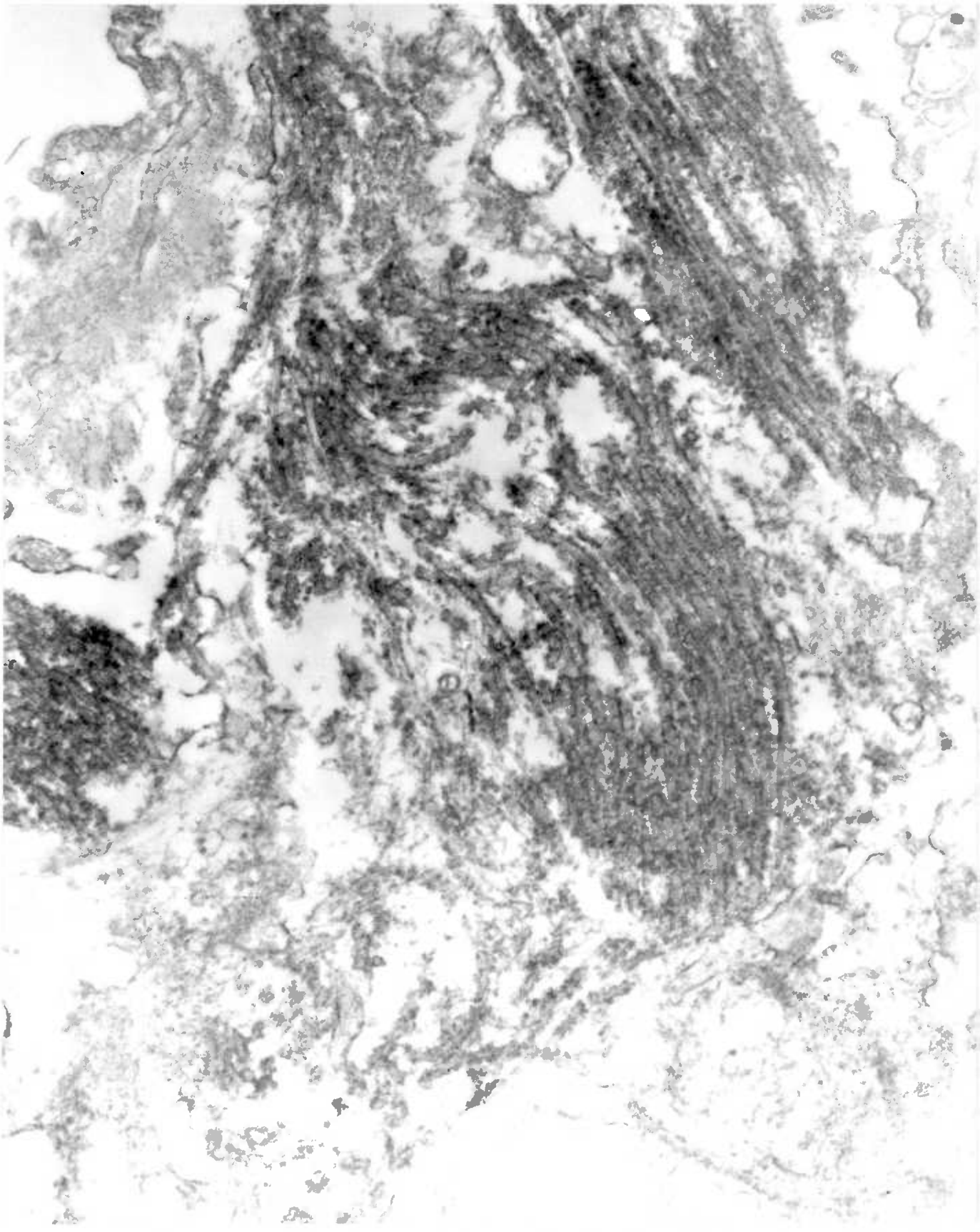


Figure IV. 19 . Electron micrograph showing higher magnification of connective tissue fibres shown in figure IV. (x32,500) The periodicity of the cross banding on the fibres suggests that they are collagen fibres. The distance between the centres of the stained parts of the fibres are approximately 3 mm (3×10^{7}Å) Magnification on this EM is 32,500 (3.25×10^{4})

Hence distance here is $\frac{3\times10^{7}}{3.25\times10^{4}}$ Å = approx. 10^{3}Å (920Å)

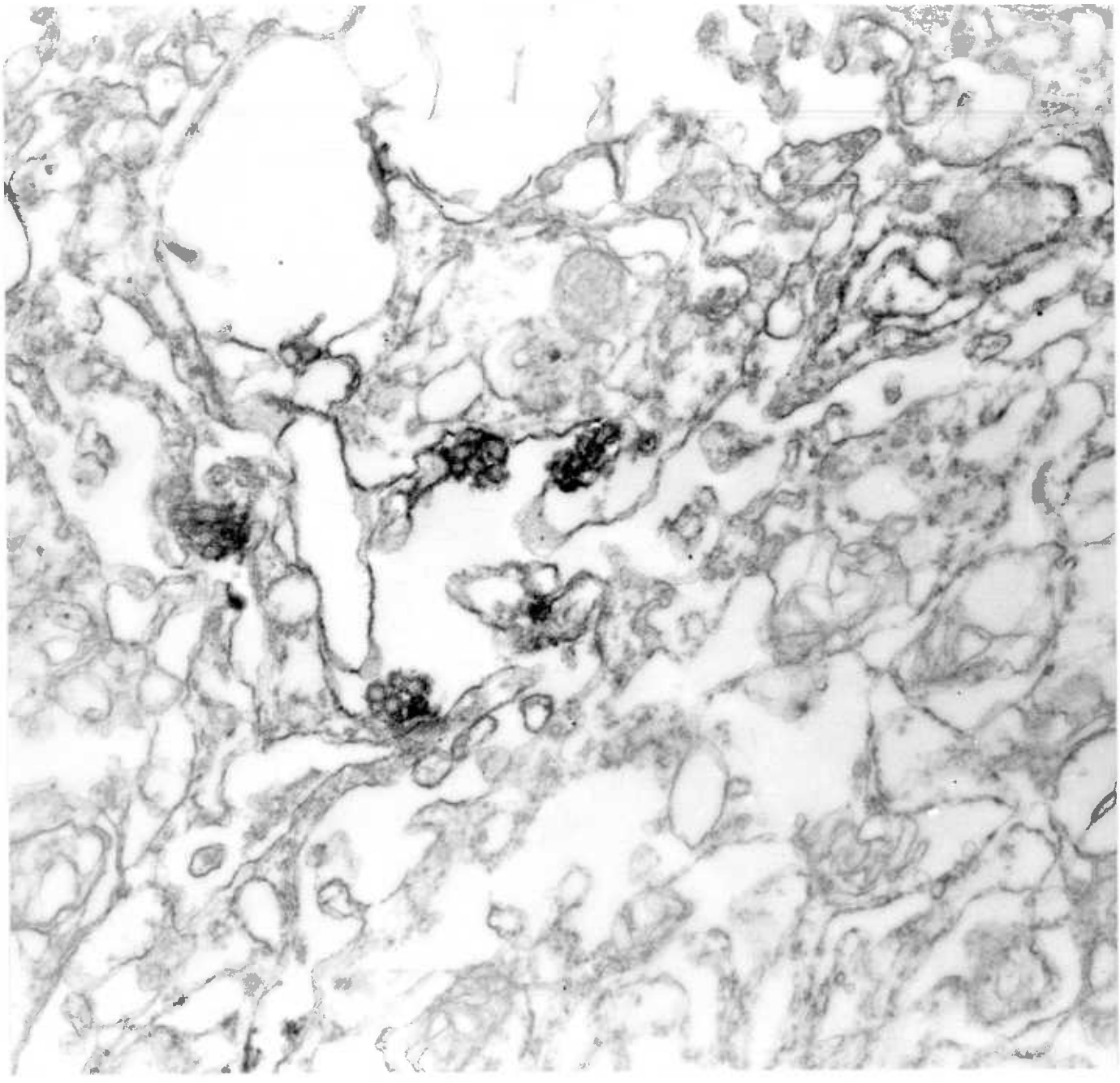


Figure IV.20 . Electron micrograph showing cross section of collagen fibres illustrated in figure IV. 19 . Note that the actual fibres are not taking up the horse radish peroxidase-complex - whereas the regions surrounding them (less mature collagen or pro-collagen(reticulin)) are.

x32,500

Absorption Studies:

Absorption of ARA positive Sera from Patients with Gluten-sensitivity with Gluten.

Of the 17 ARA positive sera tested in these absorption studies, complete removal of ARA activity was found consistently in 3 sera - one from an adult coeliac disease patient and 2 from D.H. patients (Table IV. 4). In a further 5 ARA positive sera, a distinct reduction in the intensity of staining was observed with absorption, but complete eradication of the ARA staining was never complete. In these five, it was interesting to note that the pattern of immunofluorescence staining was considerably modified by absorption. Hence, the well defined pattern was abolished leaving behind a much more diffuse connective tissue staining pattern.

In the remaining 9 of the 17 positive sera, no absorption or modification of immunofluorescent staining pattern was observed.

With the four control sera (ANF, gastric parietal cell, mitochondrial and smooth muscle antibodies positive) no diminuation or modification of immunofluorescent staining was observed, suggesting that the changes observed with ARA positive sera were not due to non-specific absorption with gluten or fraction 3.

Immunofluorescent Staining with Experimental Anti-Connective Tissue Antisera.

1. Rabbit-anti-bovine Heart Valves(BHV)

With this antiserum, immunofluorescent staining patterns very similar to that obtained with ARA positive sera from D.H. and coeliac disease patients were seen. In general however, the staining patterns were more defused and less well defined than that obtained with ARA. In addition, in the rat kidney, marked glomerular and Bowman's Capsule staining was observed. These findings suggest that in BHV a wider spectrum of connective tissue antigens are present.

	ARA +ve	Absorped	Affected	Unaffected
Childhood Coeliac Disease	7	0	2	5
Adult Coeliac Disease	4	1	1	2
Dermatitis Herpetiformis	6	2	2	2
	17	3	5	9

Controls (ANF, GPC, Mito, SM) - all unaffected by absorption procedure.

Table IV.4

Results of absorption studies of 17 anti-reticulin antibody (A.R.A.) positive sera with gluten Fraction-3.

ANF = Antinuclear factor

GPC = Gastric Parietal cell antibody

Mito = Mitochondrial antibody

SM = Smooth muscle antibody

2. Rabbit-anti-saline-insoluble non-collagenous reticulin component (NCRC) of pig (and human) kidney.

With these sera, immunofluorescent staining extremely similar to that obtained with ARA was seen. The only difference which could be pointed out distinctly was that with these antisera, more glomerular staining was seen in the rat kidney. Otherwise, all other staining was identical with that observed using ARA.

Absorption Procedures with Connective Tissue Extracts:

1. Bovine Heart Valve (BHV) Extracts.

Absorption with Crude Soluble Collagen (CSC).

Both the D.H. and ACD ARA positive sera were absorbed at all dilutions by the CSC. At the 1:16 and 1:32 dilutions there was complete removal of the ARA activity compared with the controls. At the 1:4 and 1:8 dilutions, the intensity of ARA staining was markedly diminished, though still present. The smooth muscle antibody present in the ACD serum was not removed by the absorption procedure. This suggests that the absorption with CSC for ARA was specific and suggests that common antigenic determinants are shared by CSC and the 'reticulin' responsible for the production of ARA in patients with D.H. and C.D.

Absorption with CTC Buffer Extract.

The absorptions with CTC (and CSC) using 2 ARA positive sera from D.H. at higher dilutions are shown diagramatically below.

Serum - M.McN.

Dilution	1 : 5	1 : 10	1 : 20	1 : 40
Control	++	+	+	W
CTC absorbed	+	-	-	-
CSC absorbed	+	W	-	-

++ = very strong ARA staining
+ = strong ARA staining
W = weak ARA staining
- = no ARA staining

Serum - S.T.

Dilution	1 : 5	1 : 10	1 : 20	1 : 40
Control	++	++	+	+
CTC absorbed	+	W	-	-
CSC absorbed	+	W	-	-

Result: Absorption of ARA with CTC and CSC extracts has been demonstrated in both these D.H. sera.

2. Absorption with Human Non-Collagenous Reticulin Component (NCRC).

Complete absorption or marked reduction of the ARA staining pattern was found in 6 of the 7 sera from patients with coeliac disease, and in 3 of the 4 sera from patients with dermatitis herpetiformis. The controls (sera positive for antinuclear, gastric parietal cell, smooth muscle and mitochondrial antibodies), were not affected by the incubation procedure, indicating the specificity of the absorption of ARA by NCRC.

Anti-Reticulin Antibody (ARA):

Only the 'typical' ARA immunofluorescent patterns described on page 75 will be discussed.

Dermatitis Herpetiformis, Childhood and Adult Coeliac Disease Patients.

Sera from 50 D.H. patients, 101 adult coeliac disease patients and 46 patients with childhood coeliac disease were examined in this study. All sera were initially screened at a 1:10 dilution, and positive sera titrated subsequently to a 1:80 dilution.

Of the 50 D.H. patients, 20 were on a gluten-free diet (GFD), and 30 on a normal diet. 50 of the adult coeliac disease patients were on a GFD and 51 on a normal diet. Of the 46 childhood coeliac disease patients, 20 were on a GFD and 26 on a normal diet.

ARA was detected in 11 (22%) of the 50 D.H. patients. It was found in only 2 (10%) of the 20 patients on a GFD, but in 9 (30%) of the 30 taking a normal diet (Table IV. 5). In the 2 patients on a GFD, the ARA titre was 1:40. In the 9 patients on a normal diet, ARA was present at a titre of

1:10 in two, 1:20 in two, 1:40 in two and 1:80 in three (Figure IV. 21).

In the 101 adult coeliac disease patients, ARA was present in 38 (38%). It was found in 30 (59%) of 51 patients on a normal diet, but in only 8 (16%) of the 50 taking a GFD, (Table IV. 5). In this latter group ARA was present at a titre of 1:10 in 5 patients, 1:20 in 2 patients, and 1:40 in one. In the group of patients on a normal diet, ARA was present in a dilution of 1:10 in 21, 1:2 in 2, 1:40 in 5 and 1:80 in 2 (Figure IV.21).

In childhood coeliac disease, ARA was found in 27 (59%) of the 46 patients. It was present in 22 (85%) of 26 patients on a normal diet, and in 5 (25%) of 20 taking a GFD (Table IV. 5). Of the patients on a normal diet, ARA was present in dilutions of 1:10 in 4, 1:20 in 3, 1:40 in 4 and 1:80 in 11. Of the 5 children taking a GFD who had ARA, it was present at a 1:10 dilution in 2, 1:20 in one, and 1:80 in 2 (Figure IV. 21).

Normal Controls.

Sera from 28 healthy control subjects were examined for the presence of ARA. They comprised 13 males (age range 20 - 79, average 47 years), and 15 females (age range 19 - 55, average 33 years). ARA was not detected in any of these sera.

Crohn's Disease.

This was the only group other than patients with D.H. and adult and childhood coeliac disease where ARA was found in a significant proportion of patients. It was found in 9 (24%) of 38 patients, but in low titres - 1:10 in 7 and 1:20 in 2.

Other Disorders.

534 other sera from patients with various skin, gastrointestinal, immune and other miscellaneous disorders were looked at for the presence of ARA. These comprised:

Gastrointestinal Disorders

Other malabsorption states - 35

Ulcerative Colitis - 11

Children with a variable degree of villous atrophy, thought not to be gluten induced, e.g., post-gastroenteritis malabsorption - 35

Alpha Chain disease - 4

Skin Diseases.

Pemphigus - 20

Pemphigoid - 22

Vitiligo - 25

Psoriasis - 30

Cutaneous Vasculitis - 12

Discoid and systemic lupus erythematosus - 20

Other skin disorders, including erythema nodosum, erythema multiforme, eczema, photosensitivity, drug eruptions, urticaria, necrobiosis lipoidlica and lichen sclerosus - 50

Haematological Disorders.

Adult Pernicious anaemia - 40

Nutritional megaloblastic anaemia - 10

Autoimmune haemolytic anaemia - 25

Chronic lymphatic leukaemia - 9

Myelosclerosis - 5

Immune disturbances.

Sarcoidosis - 20

Alopecia areata - 9

Myasthemia gravis - 4

Scleroderma - 10

Rheumatoid arthritis - 15

Thyrotoxicosis - 3

Fibrosing alveolitis - 1

Lipodystrophy - 1

Miscellaneous Disorders.

Fibrotic lung disease (chronic bronchitis and tuberculosis) - 20

Liver diseases (Cirrhosis, chronic active hepatitis, primary biliary cirrhosis, cryptogenic cirrhosis, infective and viral hepatitis, cholecystitis) - 55

Rickets - 2

Reticulum cell sarcoma - 1

Peptic ulceration - 1

Leg ulcer - 1

Parkinson's Disease - 1 Pregnant women - 17

Of the 534 other sera tested, ARA was detected in only 8 patients. These comprised 2 from patients with sarcoidosis, 1 patient with reticulum cell sarcoma, 1 patient with vitiligo, 1 patient with tropical sprue and in 2

pregnant women. All were at a titre of 1:10 except the patient with reticulum cell sarcoma (1:20) and the patient with vitiligo (1:80).

Effect of a gluten-free diet on the titre of ARA.

In one D.H. patient, the titre of the ARA could be followed in relation to treatment with a gluten-free diet. With treatment, the titre of the ARA fell and eventually, disappeared completely. This is illustrated diagramatically in Figure IV. 22.

Direct Immunofluorescence Studies on Skin from D.H. Patients:

80 skin biopsies from 50 D.H. patients were available for study. These 80 biopsies comprised:

15 from early erythematous skin, early blisters or peribullous skin of patients not on treatment.
37 from clinically uninvolved skin of patients controlled on dapsone alone.
15 from clinically uninvolved skin of patients not on treatment.
13 from clinically uninvolved skin of patients controlled on a gluten-free diet only.

All the 80 biopsies were studied by direct immunofluorescence for the presence of IgG, IgM and IgA. Using direct and indirect immunofluorescence, 34 biopsies from 19 patients were also tested for the presence of the C1q component of complement, 52 biopsies from 25 patients for the C3 component of complement, 10 biopsies from 10 patients for kappa and lamda light chains, and 4 biopsies from 4 patients for the presence of IgA secretory piece.

Results: Staining for IgG, IgM and IgA.

The results are tabulated in Table IV. 6.

Two main patterns of immunofluorescent staining were seen in the skin biopsies studied:-

1. Continuous homogeneous pattern:- This is shown in Figure IV. 23. A thickish band of continuous immunofluorescence is seen along the dermo-epidermal junction. Under higher magnification (as seen in Figure IV. 24), the band is seen to vary in thickness with frequent invaginations into the superficial

GROUP	TOTAL STUDIED	DIETARY STATUS		ANTI-RETICULIN ANTIBODY POSITIVE		TOTAL A.R.A. POSITIVE
		ON G.F.D.	ON NORMAL DIET	ON G.F.D.	ON NORMAL DIET	
Childhood Coeliac disease	46	20	26	5 (29%)	22 (85%)	27 (59%)
Adult Coeliac disease	101	50	51	8 (16%)	30 (60%)	38 (38%)
Dermatitis Herpetiformis	50	20	30	2 (10%)	9 (30%)	11 (22%)

Table IV.5 Incidence of anti-reticulin antibody (A.R.A.) and relationship to a gluten-free diet (G.F.D.) in patients with childhood and adult coeliac disease and with dermatitis herpetiformis.

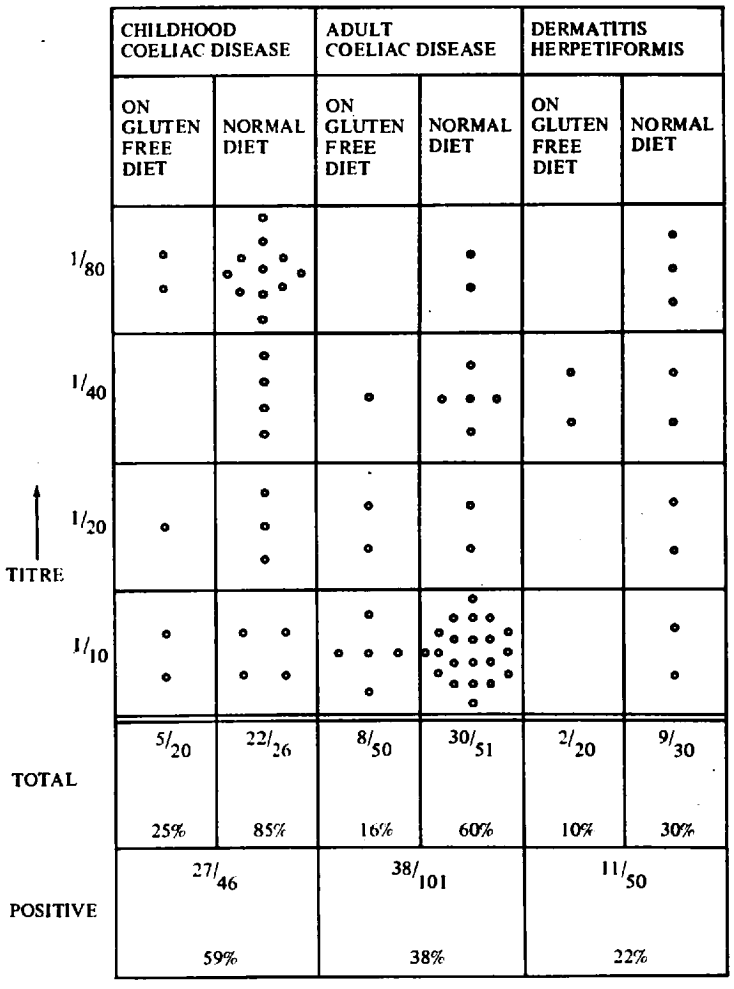


Figure IV. 21

Anti-reticulin antibody in relation to diet in childhood and adult coeliac disease and dermatitis herpetiformis.

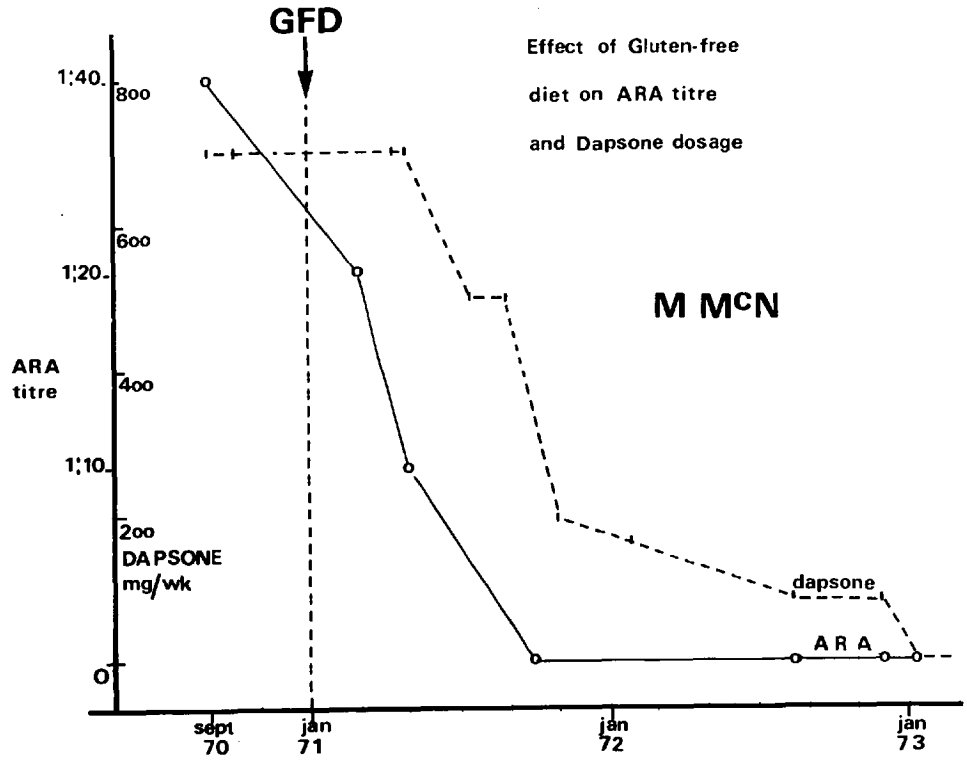


Figure IV :-22

Figure illustrating the relationship of anti-reticulin antibody titre (ARA) and dapsone dosage to a gluten-free diet (GFD) in patient Mrs. M. McN. over a period of 28 months. In January, 1974, the patient remains free of skin lesions whilst on a GFD.

dermis and dermal papillae. The band is completely continuous and there are no breaks to be seen within any one section of skin. A further example stained by the horse-radish peroxidase method (IgG class) is shown in Figure IV.33

2. Papillary Staining:- Examples of this are illustrated in Figures IV. 25 to 28, and IV. 31 and IV. 35. With this pattern of staining, the IgA deposits are confined to the dermal papillae. Very rarely, they reach down towards the region of the rete pegs of the epidermis. Two main patterns of papillary staining are seen.

(a) 'Microgrannular' pattern. (Figures IV. 25, 27, 35.)

The deposits are small, circular and resemble 'spots'. They are found very closely related to the dermo-epidermal junction. When present in large numbers they may mimic a 'continuous' pattern (Figure IV. 27) or form large homogeneous deposits located in the dermal papillae (Figures IV. 31 and 35.).

(b) 'Fibrillar' pattern. (Figure IV. 26).

With this staining pattern, the IgA deposits are present as definite fibrils located within the dermal papillae. They tend to reach further down into the center of the dermal papillae than the microgrannular deposits.

In the majority of skin biopsies combinations of both 'microgrannular' and 'fibrillar' deposits are seen together. (Figure IV. 28). In a few however, only the 'microgrannular' or 'fibrillar' pattern was present on its own.

In 3 biopsies of early bullous lesions, the immunoglobulin deposits were scattered in the sub-epidermal blisters and showed no consistent pattern (Figure IV. 37). For convenience, these deposits will be categorised under the 'papillary' pattern.

Immunoglobulin Class.

Patients. IgA was present in all 50 patients studied (Table IV. 6). IgM was present in addition to IgA in 7 patients, and IgG in addition to IgA in 3 patients. In one patient both IgM and IgG were found as well as IgA.

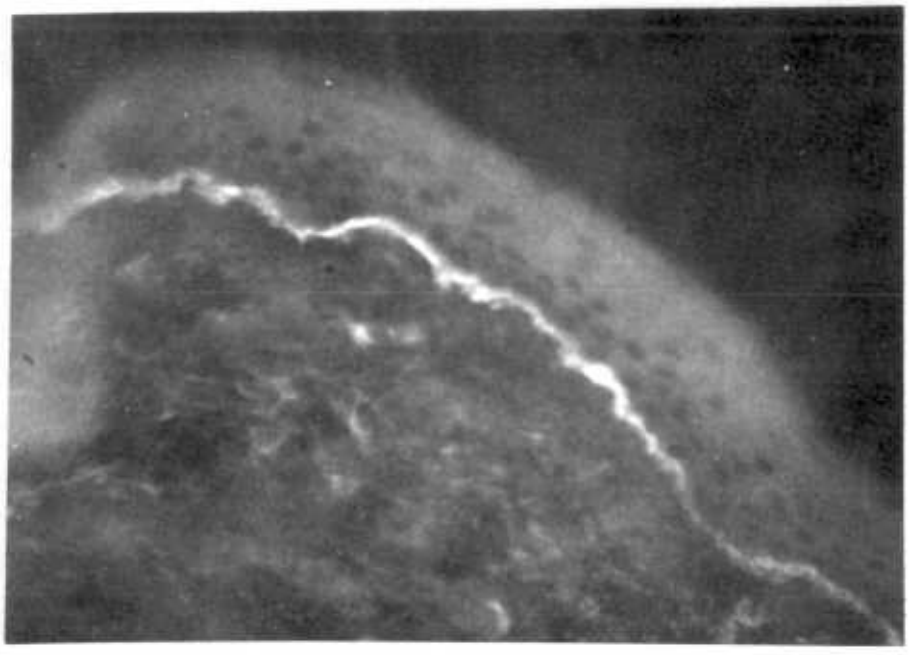


Figure IV.23 : Uninvolved D.H. skin showing a homogeneous 'continuous' pattern of IgA staining along the dermo-epidermal junction.

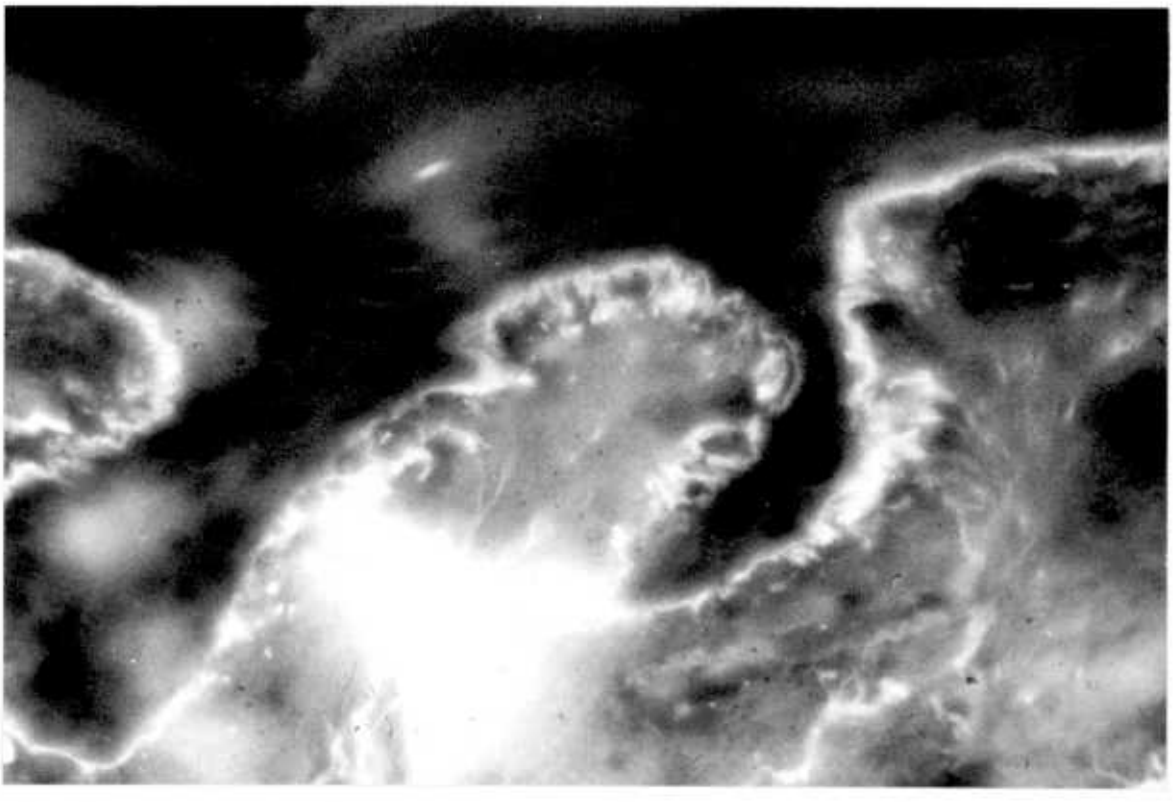


Figure IV. 24 : Uninvolved D.H. skin showing a 'continuous' pattern of staining with FITC-anti-human C3. This specimen of skin also showed similar deposits of IgG, IgA and C1q.

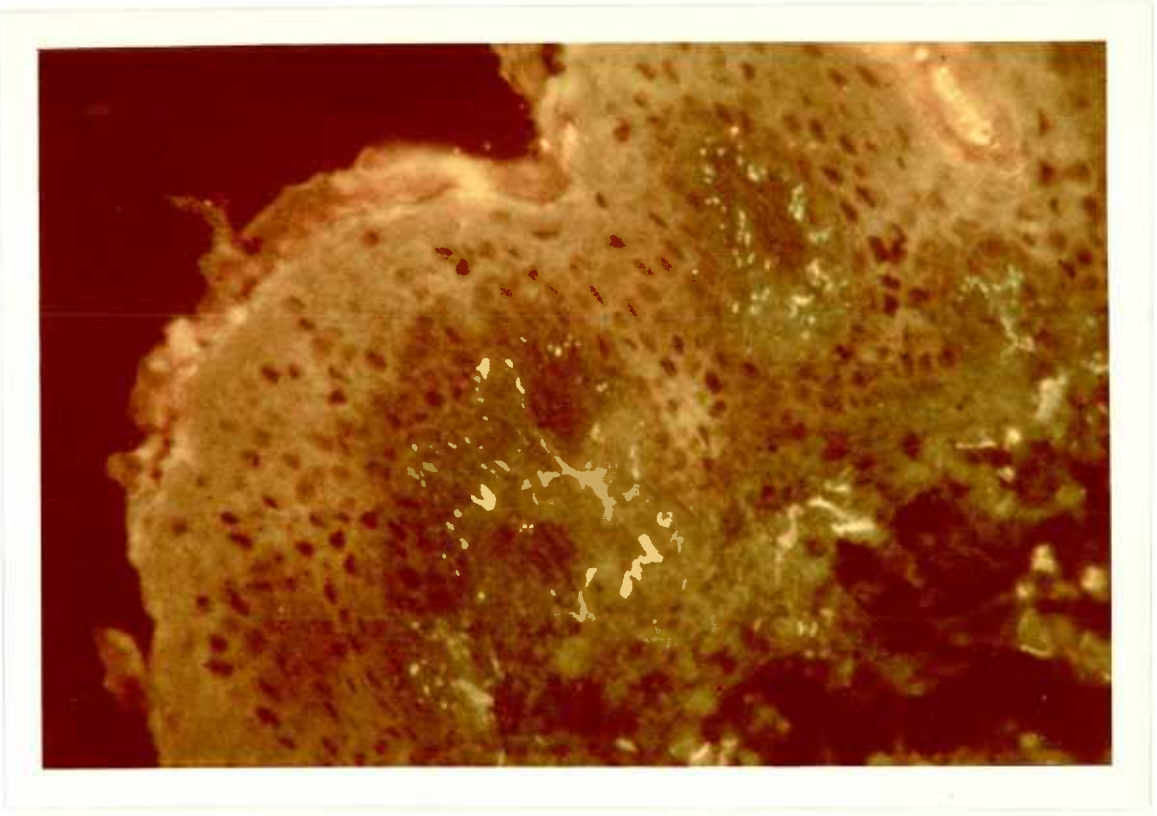


Figure IV.25 : Uninvolved D.H. skin showing 'Micrograniular' IgA deposits in the dermal papillae.

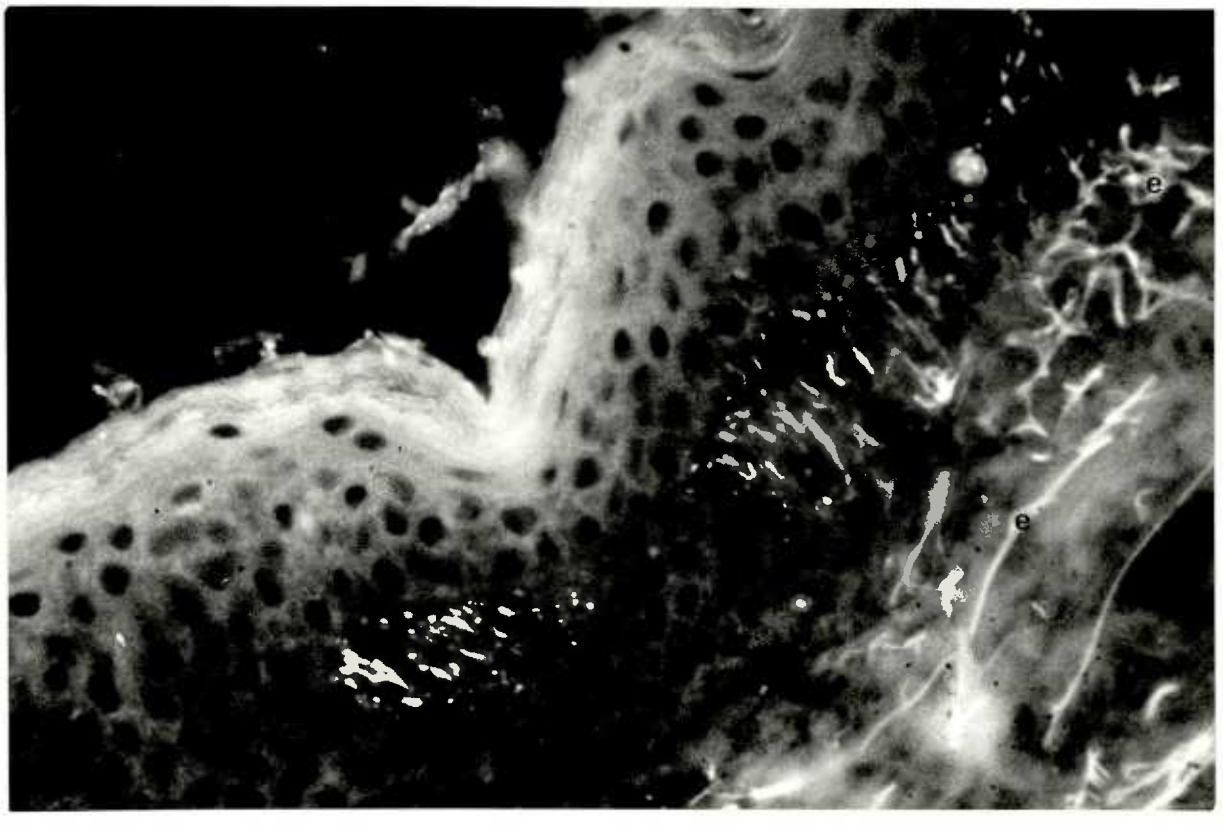


Figure IV.26 : Uninvolved D.H. skin showing 'fibrillar' IgA deposits in the dermal papillae. There is also autofluorescence of elastic fibres (e) deeper in the dermis.

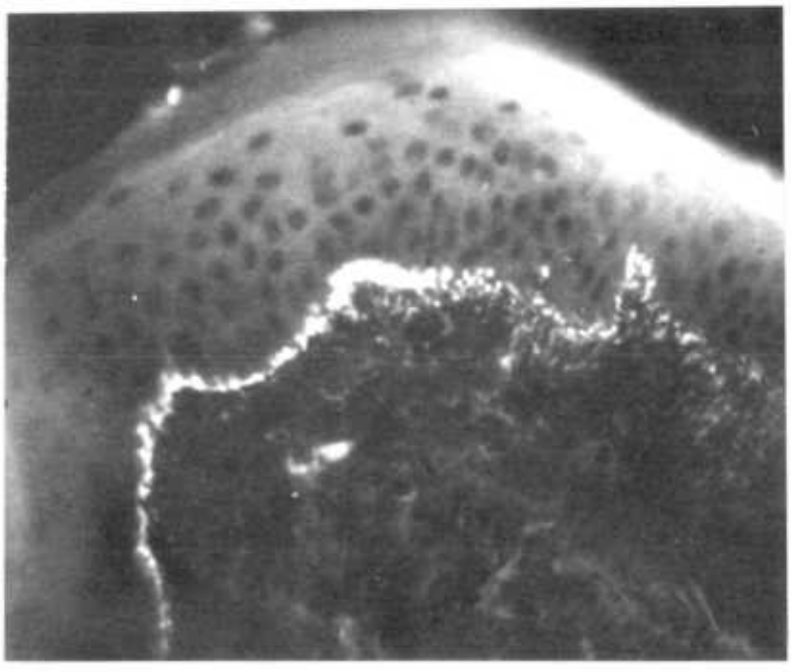


Figure IV . 27

Uninvolved D.H. skin showing dense deposits of 'microgranular' IgA along the dermo-epidermal junction.

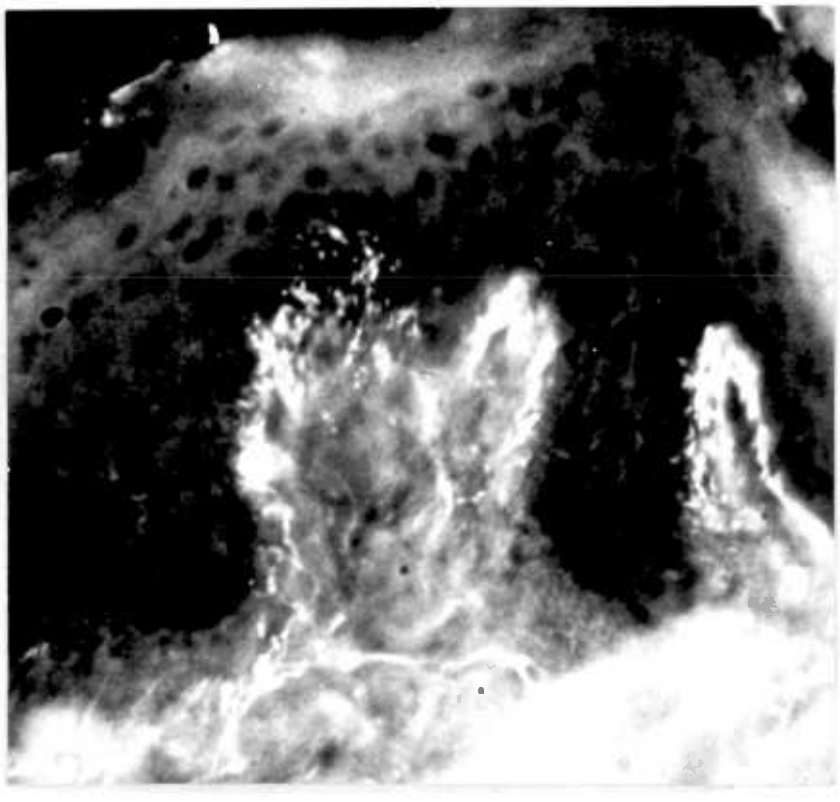


Figure IV . 28

Uninvolved D.H. skin showing a mixture of 'fibrillar' and 'microgranular' IgA deposits in the dermal papillae.

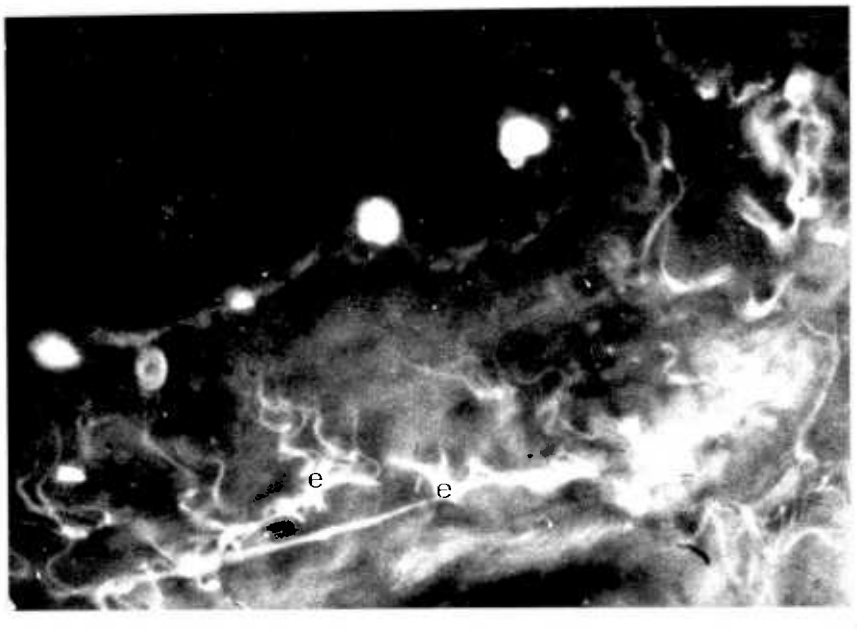


Figure IV . 29

Skin from normal control stained with FITC-sheep-anti-human IgM showing markedly fluorescent 'globules' situated at the dermo-epidermal junction.

E = autofluorescent elastic fibres.

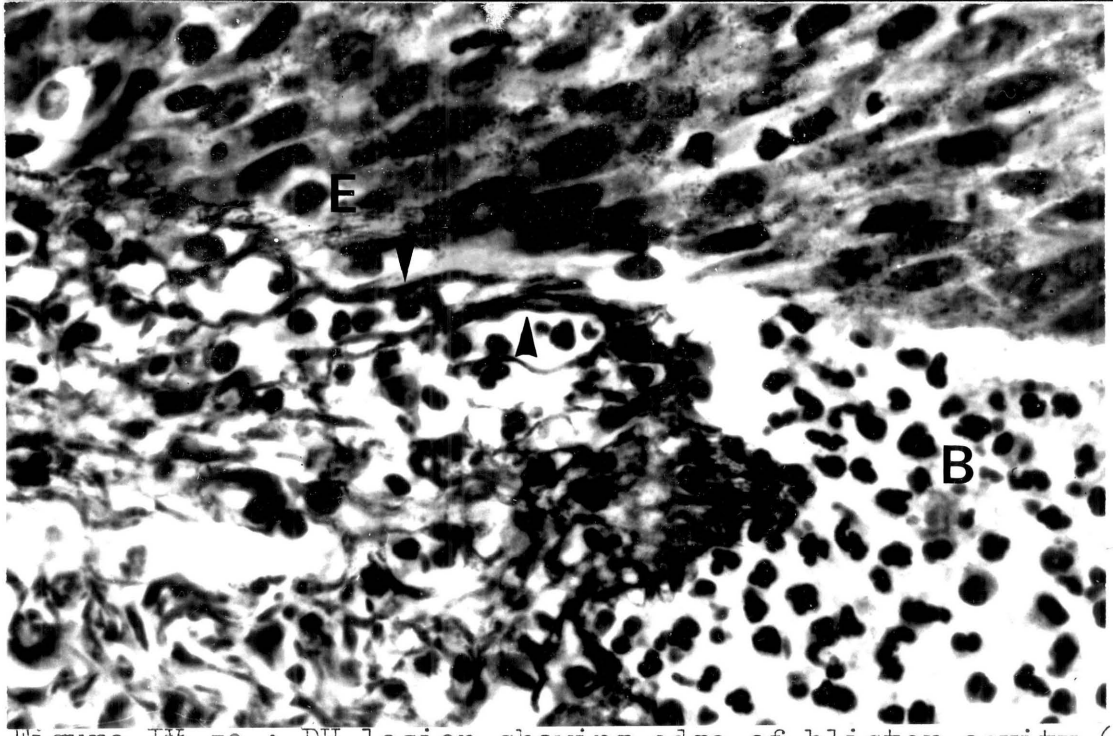


Figure IV. 30 : DH lesion showing edge of blister cavity (B). Gomori silver stain showing reticulin fibres (arrows) seperating away from epidermis (E).

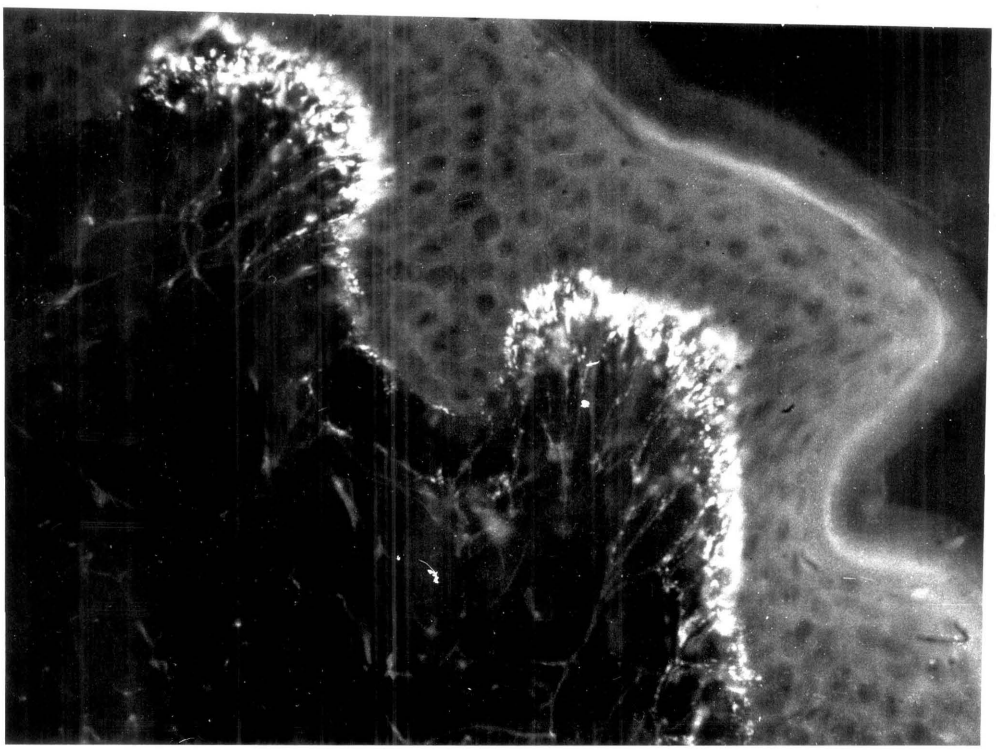


Figure IV. 31 : DH uninvolved skin. Direct immunofluorescence showing furthur examples of IgA deposition in the dermal papillae.

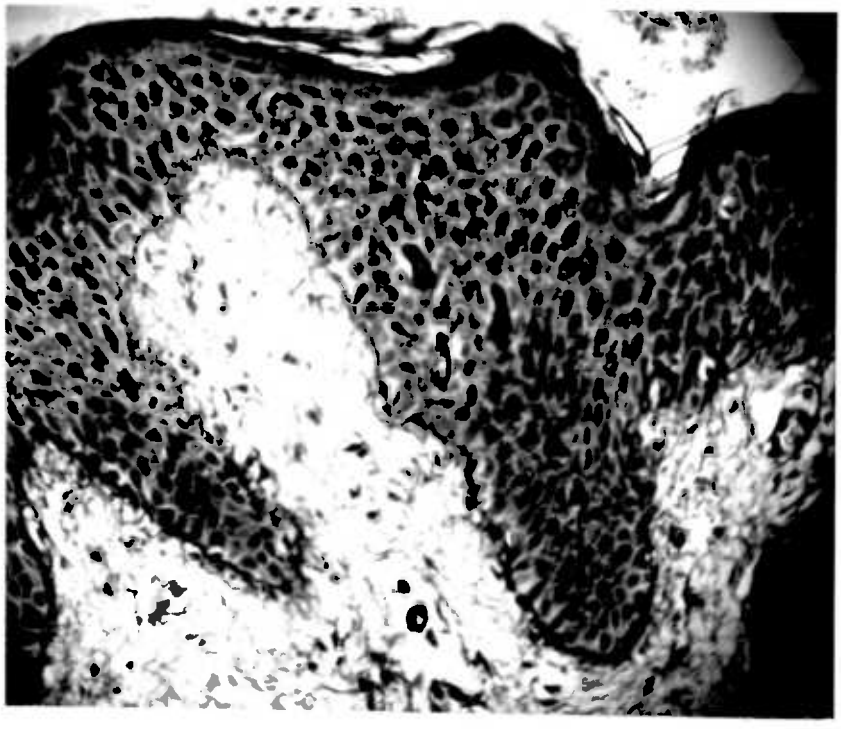


Figure IV.32 : DH uninvolved skin - Gomori silver stain showing reticulin fibres localized to dermo-epidermal junction.

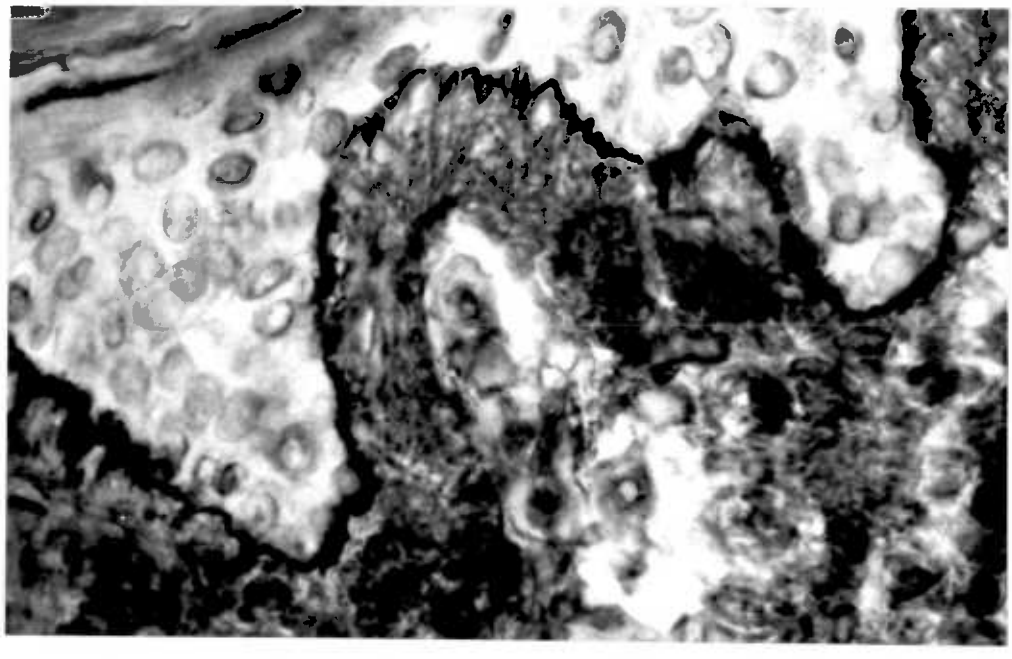


Figure IV. 33 : Uninvolved DH skin - Horse-radish peroxidase labeled-anti-human IgG showing dense band of immunoglobulin deposition as a 'continuous'line along the dermo-epidermal junction.

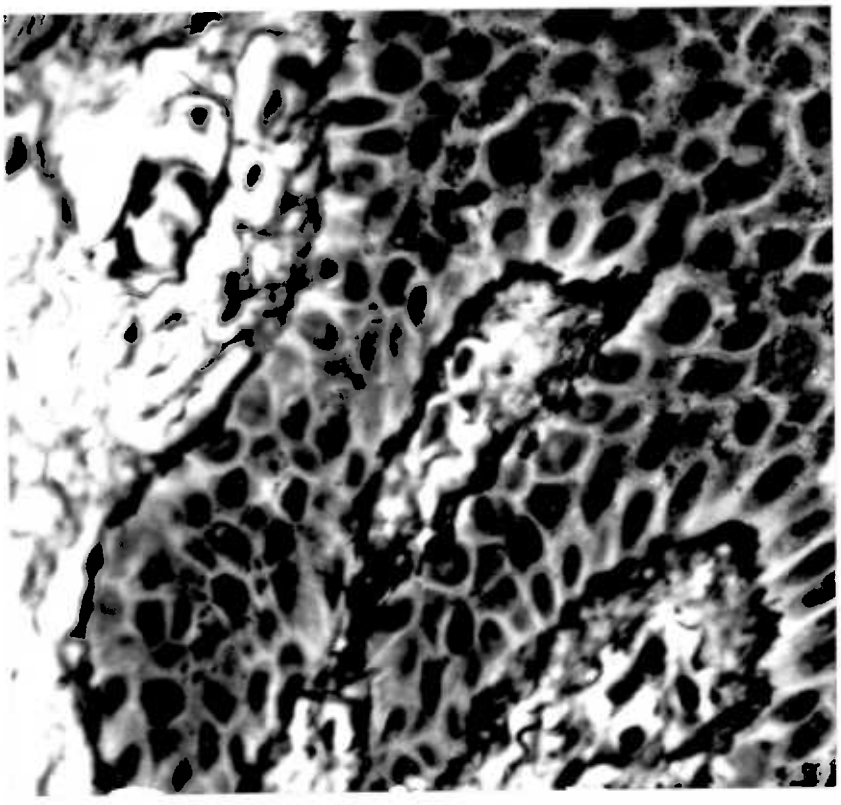


Figure IV. 34
DH uninvolved skin Gomori silver stain showing HP view of reticulin fibres in dermal papillae at dermo-epidermal junction.

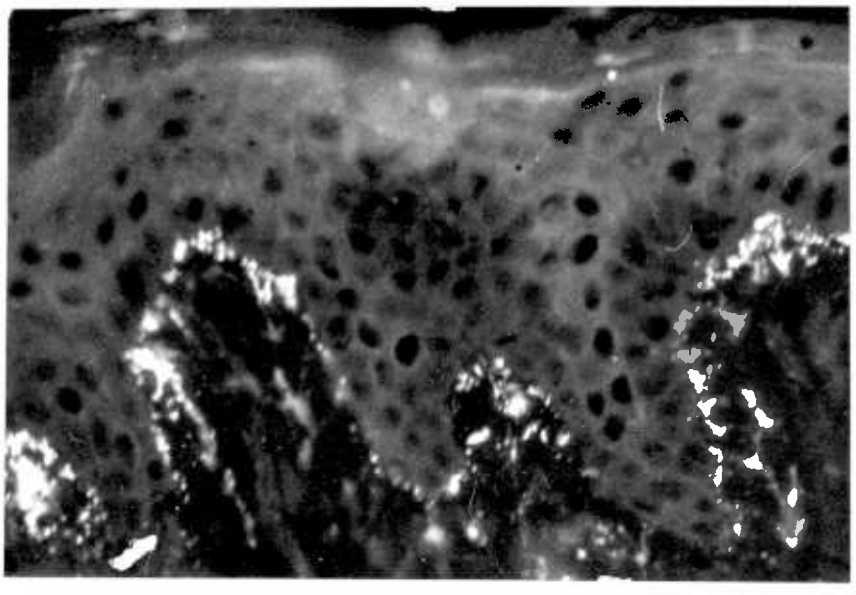


Figure IV. 35
DH uninvolved skin - direct immunofluorescence showing IgA deposits in similar position in dermal papillae.

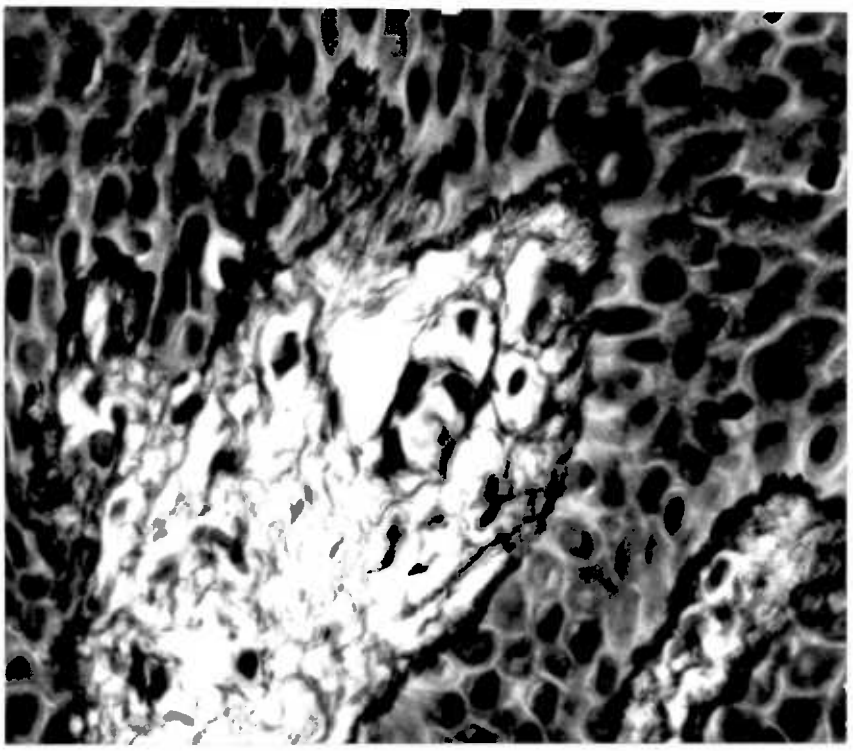


Figure IV. 36
Early DH lesion showing microabscess - Gomori silver stain showing reticulin fibres.

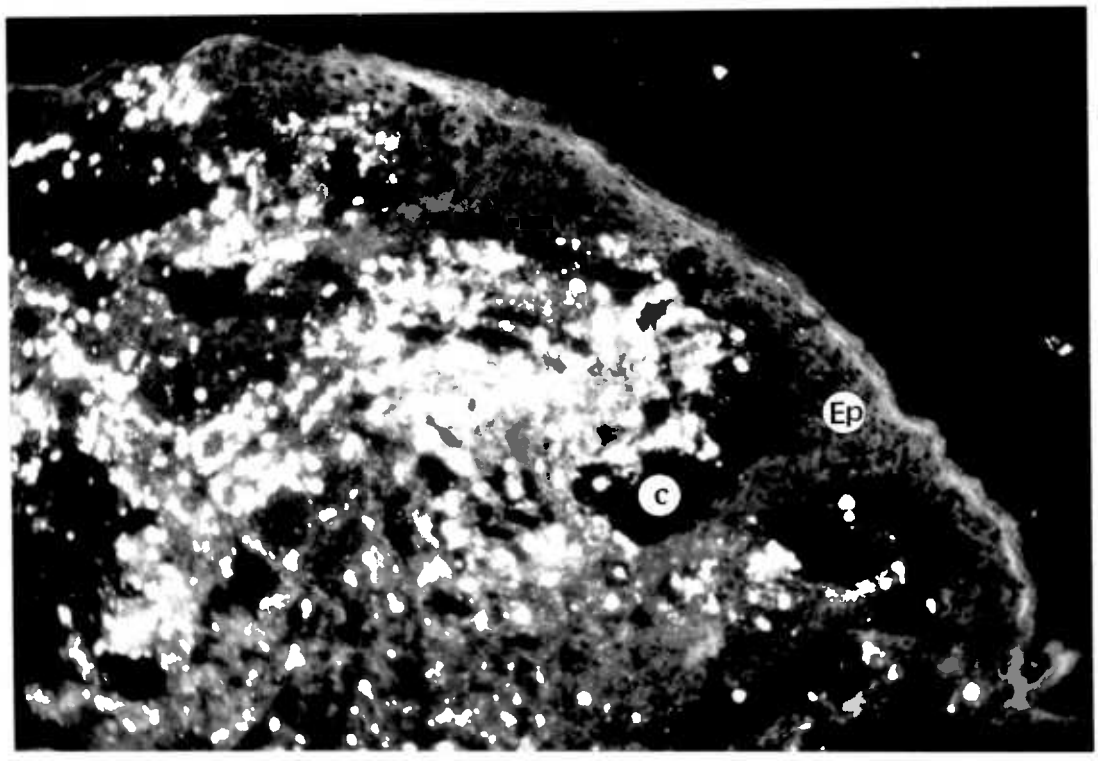


Figure IV.37 : Early D.H. blister showing dense IgA deposits in the subepidermal blister cavity.
Ep = epidermis c = blister cavity.

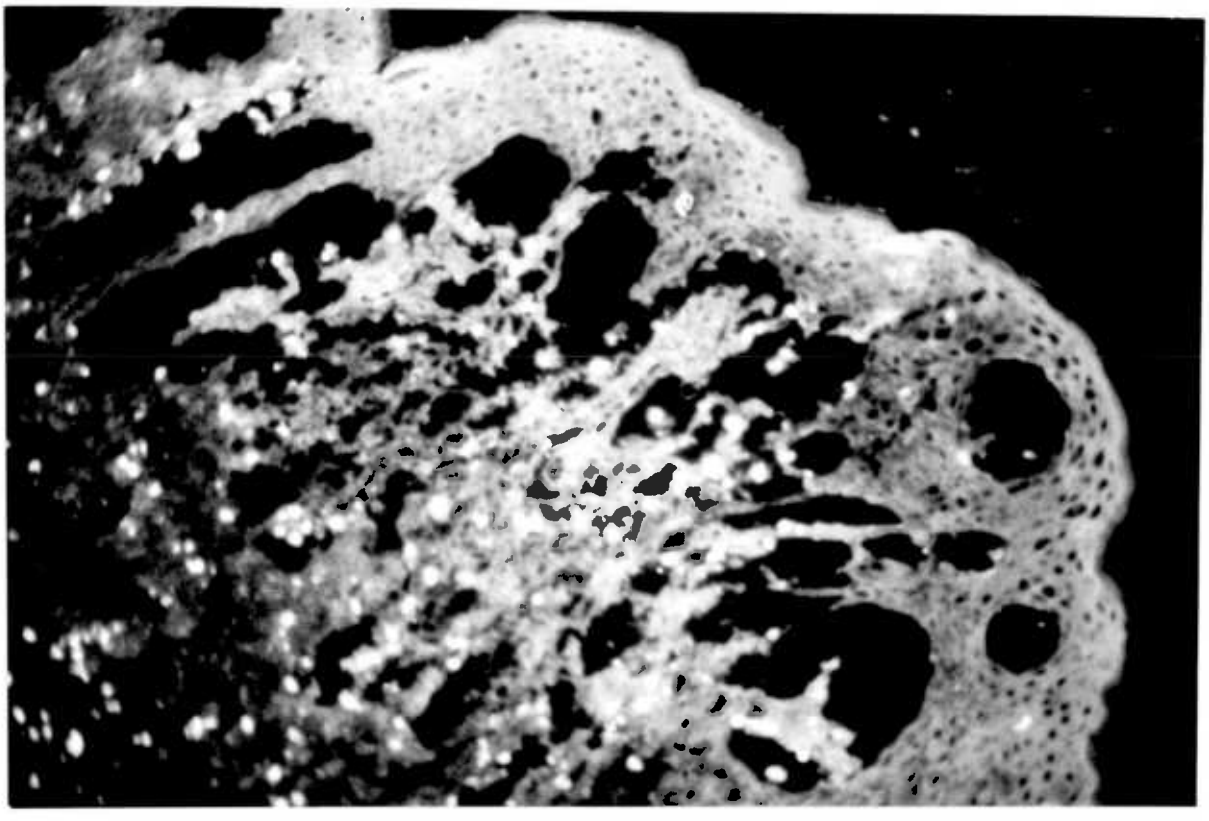


Figure IV.38 : Same specimen of D.H. blister as above stained with FITC-anti-human C3 showing deposits in the dermis in the same distribution as the IgA. Staining with anti-C1q was negative. There is marked non-specific staining of the epidermis.

Skin Biopsies. IgA was detected in 78 of the 80 skin biospies; IgM in 8, and IgG in 3 (Table IV. 7). In the 2 biopsies in which no IgA was detected, neither IgG nor IgM were found. In 6 of 7 biopsies which showed the presence of IgM, and in 2 of the 3 biopsies with IgG, this was in addition to IgA. In one biopsy all three classes of immunoglobulin (IgA, IgG, IgM) were present. IgM or IgG were never detected as the sole class of immunoglobulin in the absence of IgA.

Pattern of Immunoglobulin Deposits.

The patterns of immunoglobulin deposition in the positive skin biopsies were:-

Papillary only. In 68 of the 78 positive biopsies immunoglobulins were present in the papillary form. In 60 of the 67 biopsies IgA was present alone, in 6 IgM and IgA were present, and in one IgG and IgA (Table IV. 8).

Continuous only. This was the pattern in 9 of the biopsies. In 8 of the 9, IgA was the only class of immunoglobulin present, and in one IgG was found in addition to IgA (Table IV. 8).

Papillary & Continuous. In 2 biopsies, both the continuous and papillary patterns of immunoglobulin deposition were present. In addition, it was found that in both biopsies the class of immunoglobulin was different in the papillary and continuous deposits. In the one biopsy there was continuous IgA and papillary IgM, and in the other there was continuous IgM and IgG, and papillary IgA (Table IV. 8).

Multiple Biopsies of Uninvolved Skin at Different Times.

Fifteen patients had 2 biopsies from uninvolved skin on two different occasions (Table IV. 9). In 9 of the 15 patients the class of immunoglobulin and pattern of immunofluorescent staining were the same. All 9 of these patients had IgA deposits showing only the papillary pattern. Of the remaining 6 patients, 2 failed to show any immunoglobulin deposits on the first biopsy, but showed papillary IgA on the second biopsy. Two other patients showed

papillary IgA alone on one biopsy and papillary IgA and IgM together on the other biopsy. The remaining two patients showed differences in both the class and pattern of immunoglobulin deposition. Thus, one patient showed papillary IgA and continuous IgG and IgM on the first biopsy, but only papillary IgA on the second biopsy. The other patient showed papillary IgM and continuous IgA on the first biopsy, but only continuous IgA on the second.

Comparison of Uninvolved Skin and Early Lesions.

Immunoglobulin deposits were found in all the 15 biopsies from early lesions. In 14 of these 15 patients, the class and pattern of immunoglobulin deposition was the same as that found in the uninvolved skin. In 10 of the 15 patients only IgA in a papillary pattern was found in both the uninvolved and early lesions (Table IV. 10). In 3 other patients IgA in a continuous pattern was present in both involved and uninvolved skin. Of the remaining 2 patients, one had IgA and IgM in a papillary pattern in both the involved and uninvolved skin, and in the patient in whom there was a difference between involved and uninvolved skin, IgA in a papillary pattern was present in the uninvolved skin, whilst in the involved skin both IgA and IgM were present in a papillary pattern.

Pattern and Class of Immunoglobulin Deposition in Uninvolved Skin in Relation to Treatment.

In all there were 65 biopsies from the uninvolved skin. Of these, 36 biopsies were from patients taking dapsone, 14 from patients whose eruption was controlled with a gluten-free diet, and 15 from patients not receiving any therapy. There appeared to be no significant difference in the class or pattern of immunoglobulin deposition in relation to treatment. IgA alone in a papillary pattern was the most common finding in all three groups, being present in 30 of the 36 patients taking dapsone, 10 of the 14 patients taking a gluten-free diet, and 10 of the 15 patients not receiving any therapy (Table IV. 11). IgA in association with IgG or IgM, or both, was present in

2 of the patients taking dapsone, 4 of the patients taking a gluten-free diet, and one of the patients not receiving any therapy. The numbers involved are too few to allow any statistical comparison. IgA in a continuous pattern was found in 3 patients taking dapsone, in one patient taking a gluten-free diet, and in 3 patients not receiving any therapy. Once again however, the numbers are too small for statistical analysis. It would appear however, that there is no correlation between the class and pattern of immunoglobulin deposition and therapy.

Serological Studies in Patients with the Continuous Pattern of Immunoglobulin Deposition.

In the 6 patients who showed the continuous pattern of immunoglobulin deposition in one or more of their biopsies, anti-reticulin antibody was present in one. No circulating antibodies to basement membrane or epidermal intercellular cement substance were detected.

Comparison of IgA Deposits Detected by Direct Immunofluorescence to Histological Morphology.

Formalin fixed biopsies of skin taken from the same sites as those for immunofluorescence were precessed histologically using the following stains.

1. Haematoxylin and eosin.
2. Gomori silver stain for reticulin (Gomori 1937).
3. Van Giesen stain for collagen and elastin (9 parts saturated aqueous picric acid to 1 part of 1% acid fuchsin).

Histologically, the IgA deposits found by immunofluorescence corresponded best to the reticulin fibres seen along the dermo-epidermal junction below the basement membrane (Figures IV. 30, 32, 34, 36). No correlation was evident with collagen or elastic fibres, or any particular cell type. Unlike the reticulin histological stain however, the immunofluorescent IgA deposits of the papillary type did not extend down to involve the area of the dermo-epidermal junction around the rete pegs of the epidermis. There was however, a close relationship with the 'continuous' immunofluorescent IgA deposits.

Table IV. 6 (table on following two pages)

Direct immunofluorescent studies on involved and uninvolved uninvolved skin of 50 patients with dermatitis herpetiformis.

Dap.	=	Dapsone
Inv.	=	Involved skin
Uninv.	=	Uninvolved skin
GFD	=	Gluten-free diet

For direct immunofluorescence

-	=	negative
+	=	positive 'papillary' pattern of immunofluorescence
+C	=	positive 'continuous' pattern of immunofluorescence

...../cont'd

No.	NAME	Ig Class			THERAPY			
		IgG	IgM	IgA	On Dap.	Off Dapsone		GFD only
						INV.	UNINV.	
1	B P	-	-	+		+		
		-	-	+			+	
2	M H	-	-	+	+			
		-	-	+		+		
		-	-	+			+	
3	T P	-	-	+				+
4	D H-H	-	-	+				+
5	S T	+C	-	+C	+			
6	R S	-	+	+				+
7	S I	-	-	+	+			
8	L H	-	-	+	+			
9	M H	-	+	+				+
		-	-	+				+
10	A T	-	-	+	+			
		-	-	+		+		
		-	-	+			+	
11	G E	-	-	+	+			
12	E P	-	+	+	+			
13	G C	-	-	+				+
14	T P	-	-	+	+			
		-	-	+		+		
		-	-	+			+	
15	E L	-	-	+	+			
16	C C	-	-	+	+			
17	G J	+C	+C	+				+
		-	-	+				+
18	T E	-	-	+	+			
		-	-	+		+		
19	G F	-	-	+		+		
		-	-	+			+	
20	M McN	-	-	+	+			
21	M P	-	-	+	+			
		-	-	+		+		
		-	-	+			+	
22	M R	+	-	+				+
23	J L	-	-	+	+			
		-	-	+				+
24	E R	-	-	+	+			
25	F W	-	-	+C	+			
26	J H	-	-	+	+			

no.	NAME	Ig Class			THERAPY			
		IgG	IgM	IgA	On Dap.	Off Dapsone		GFD only
						INV.	UNINV.	
27	T R	-	-	+	+			
		-	-	+		+		
		-	-	+			+	
28	V W	-	-	+	+			
		-	-	+		+		
		-	-	+			+	
29	W Z	-	-	-	+			
		-	-	+	+			
30	S B	-	-	+	+			
		-	+	+		+		
		-	-	+			+	
31	E M	-	-	+	+			
32	A S	-	-	+	+			
33	M E	-	+	+C	+			
		-	-	+C		+		
		-	-	+C			+	
34	R J	-	-	+	+			
35	D M	-	-	+	+			
		-	-	+		+		
		-	-	+			+	
36	S P	-	-	+	+			
		-	+	+		+		
		-	+	+			+	
37	E E	-	-	+C	+			
38	K C	-	-	+	+			
39	P P	-	-	+	+			
40	P B	-	-	+	+			
41	T C	-	-	+C		+		
		-	-	+C			+	
42	J S	-	-	-			+	
		-	-	+	+			
43	M G	-	-	+	+			
44	L S	-	-	+C		+		
		-	-	+C			+	
45	J K	-	-	+	+			
46	A B	-	-	+	+			
47	D W	-	-	+	+			
48	D H	-	-	+				+
49	A S	-	-	+	+			
50	A G	-	-	+	+			

	IgA alone	IgA + IgM	IgA + IgG	IgA + IgM + IgG	No Ig.	Total
Patients	41	6	2	1	0	50
Biopsies	68	7	2	1	2	80

Table IV 7 :

Class of immunoglobulins in 80 skin biopsies of 50 D.H. patients.

Ig. = immunoglobulin

	IgA alone	IgA + IgM	IgA + IgG	IgA + IgM + IgG	Total
Papillary alone	60	6	1	0	67
Continuous alone	8	0	1	0	9
Papillary & Continuous	0	1*	0	1±	2

* Papillary IgM
Continuous IgA

± Papillary IgA
Continuous IgM + IgG

Table IV. 8 : Patterns of Immunoglobulin Deposits.

Number of Patients.	1st. Biopsy	2nd. Biopsy
9	IgA (P)	IgA (P)
2	0	IgA (P)
1	IgG (C) + IgM (C) IgA (P)	IgA (P)
1	IgM (P) + IgA (P)	IgA (P)
1	IgM (P) + IgA (C)	IgA (C)
1	IgA (P)	IgM (P) + IgA (P)

Table IV. 9 :

Direct immunofluorescent results in repeat biopsies from uninvolved skin at different times in 15 D.H. patients.

P = Papillary pattern

C = Continuous pattern

No. of Patients	Uninvolved Skin	Early Lesions
10	IgA (P)	IgA (P)
3	IgA (C)	IgA (C)
1	IgA (P) IgM (P)	IgA (P) IgM (P)
1	IgA (P)	IgA (P) IgM (P)

Table IV. 10 :

Comparison of immunoglobulin deposits in involved and uninvolved skin in 15 D.H. patients.

P = Papillary pattern
C = Continuous pattern

Treatment	Pattern of Ig deposit	IgA	IgA + IgM	IgA + IgG	IgA + IgM + IgG
Dapsone	Papillary	30/36	1/36*	Nil	Nil
	Continuous	2/36		1/36	Nil
G.F.D.	Papillary	10/14	2/14	1/14	1/14±
	Continuous	Nil	Nil	Nil	
Nil	Papillary	10/15	1/15	Nil	Nil
	Continuous	3/15	Nil	Nil	Nil

* Papillary Ig
Continuous IgA

± Papillary IgA
Continuous IgM + IgG

Table IV. 11 : Class and pattern of immunoglobulin deposits in the uninvolved skin in relation to treatment.

Interpretation of this Correlation. These comparative studies show that the IgA deposits detected by immunofluorescence are very similar in situation to the reticulin fibres detected by the Gomori silver stain. Whilst an absolute correlation cannot be assumed using only these techniques of direct comparison at light level, it is suggested that these IgA deposits are located in the reticulin fibres of the dermis.

Specificity of IgA Deposits.

The specificity of the IgA deposits, i.e., their identification as true immunoglobulin deposits containing α-heavy chain, is suggested by the following:-

(i) In most of the D.H. biopsies tested, IgA was the only immunoglobulin detected. The majority of these biopsies were also tested against anti-IgG, anti-IgM and anti-C3 and goat-anti-rabbit FITC labelled conjugates but gave negative results with these antisera. This suggests that the IgA deposits found are not due to the non-specific uptake of FITC molecules in the skin biopsies.

(ii) The FITC labelled antisera used have undergone specificity tests using smears of mono-clonal IgA myeloma (Hijmans et al. 1969 - see Table III. 3).

(iii) Using 'blocking' experiments (page 35) in 12 D.H. skin biopsies which only had IgA positive deposits, prior incubation with unconjugated anti-human IgA abolished the ability of the FITC labelled anti-IgA conjugate to give positive results on subsequent incubation with the same skin biopsies. This suggests that the specific receptor sites in the skin biopsies for anti-IgA have been blocked by the first incubation, and removed the ability of the FITC labelled anti-IgA used subsequently to react to the same receptor sites.

Initial incubation of the same biopsies with unconjugated anti-IgG or anti-IgM however, did not abolish the ability of subsequent incubation with FITC anti-IgA to give positive fluorescence. This is further support for the specificity of the IgA deposits in the skin biopsies being true immunological phenomenon.

C3 Deposits.

In this study, 52 skin biopsies from 25 patients were examined for C3 deposits using either direct immunofluorescence (Hyland FITC-anti-human C3), or by indirect immunofluorescence (rabbit-anti-C3 followed by FITC labelled goat-anti-rabbit immunoglobulin). The 52 biopsies comprised:-

19 taken from patients controlled on dapsone.
13 of involved skin of patients on no treatment.
12 of uninvolved skin of patients on no treatment.
8 biopsies of patients controlled on a gluten-free diet only.

The results of the study are shown in Table IV. 12.

All 52 biopsies had IgA deposits. 26 (50%) of the 52 had C3 deposits as well. The highest incidence of C3 positivity was found in the involved skin of patients on no treatment (12 of the 13 biopsies).

The pattern of C3 staining was generally either 'continuous' or 'papillary' and corresponded in all cases to the pattern of IgA staining.

C3 and C1q deposits in D.H. biopsies and relation to IgA, IgG and IgM deposits.

34 D.H. skin biopsies were examined in this study. All the biopsies were tested for the presence of IgA, IgM, IgG and the C3 and C1q components of complement. The C3 deposits were tested using a Hylands goat-anti-human C3 (Bic/Bia) FITC labelled conjugate. For detection of C1q, a 2 step (indirect) sandwich technique - (a) incubation with unconjugated rabbit-anti-C1q, followed by (b) goat-anti-rabbit FITC was used. The 34 D.H. biopsies studied comprised:

9 biopsies of involved skin from patients on no treatment.
9 biopsies of uninvolved skin from patients on no treatment.
8 biopsies of skin from patients controlled on dapsone.
8 biopsies of skin from patients whose rashes were controlled by taking a gluten-free diet.

Results.

IgA was found in all 34 biopsies. The pattern of staining was either 'papillary' or 'continuous' (for details see Table IV. 13).

In 9 biopsies, IgM deposits were present, and in 3, IgG (Table IV. 13).

In 16 (47%) of the 34 biopsies, C3 deposits were present, whilst C1q deposits were only found in 3 (9%) of the 34 biopsies. Where present, the

Nature of Skin Biopsy.	No. of Biopsies	C3 Positive
Patients on dapsone.	19	8
Involved skin - patients on no treatment.	13	12
Uninvolved skin - patients on no treatment.	12	3
Patients controlled on G.F.D. alone.	8	3
TOTAL	52	26(50%)

Table IV . [12]

C3 deposits in 52 skin biopsies from 25 D.H. patients:- relationship to therapy of patient and nature of skin biopsy. (G.F.D. = gluten-free diet).

pattern of C3 and C1q deposition was similar to the IgA immunofluorescent staining seen in the same biopsy.

Relation of C3 and C1q positivity to nature of skin biopsy.

The positivity of C3 and C1q was related to the type of skin biopsy taken (i.e., involved or uninvolved skin, patient on or off depsone, or whether on a GFD only.) The results are shown in Table IV. 13.

It can be seen that the highest incidence of C3 deposits was found with the involved skin of patients on no treatment (8 out of 9 biopsies). In the uninvolved skin of patients on no treatment, C3 was found in only 3 of 9 biopsies.

In the skin of patients whose rash was controlled by dapsone, C3 was found in 2 of 8 patients. C1q was also found in the same two patients. In the biopsies from patients whose rash was controlled by a gluten-free diet, C3 was present in 3 of the 8 biopsies. C1q was present in one of these 3 biopsies.

In all the 3 biopsies showing C1q deposits, either IgG or IgM deposits were also present in addition to IgA. Thus, 2 biopsies had IgM and one IgG deposits.

The pattern of immunofluorescent staining of the C3 and C1q deposits were generally similar to that obtained with the IgA deposits (Figures IV. 37 and 38).

Kappa and Lamda light chain deposits.

In this study, 10 skin biopsies from 10 D.H. patients who had only IgA deposits in their skin, were examined using FITC labelled anti-Kappa, and anti-Lamda (Bence-Jones protein) conjugates.

Of the 10 biopsies, Kappa chains were found in 4 and Lamda chains in 5. In 4 biopsies, Kappa and Lamda chains were found together in the same biopsy.

IgA-secretory piece.

This was looked for in 4 biopsies showing IgA deposits from 4 D.H. patients. An indirect immunofluorescent technique was used. After initial incubation with sheep-anti-human colostral secretory IgA, FITC rabbit-anti-sheep

Table IV. 13 34 Skin Biopsies from 19 Patients with D.H. : Relation of C_3 deposits to IgG, IgM, IgA and C_1q deposits.

Treatment		Name	IgG	IgM	IgA	C_3	C_1q
On dapsone	1	M.H.	–	–	+	–	–
8 biopsies	2	S.T.	+C	–	+C	+C	+C
	3	S.I.	–	–	+	–	–
	4	E.P.	–	+	+	–	–
	5	M.P.	–	–	+	–	–
	6	E.R.	–	–	+	–	–
	7	S.B.	–	–	+	–	–
	8	M.E.	–	+C	+C	+C	+C
			1	2	8	2	2
No treatment	9	B.P.	–	–	+	+	–
Involved	10	M.H.	–	–	+	+	–
	11	A.T.	–	–	+	+	–
9 Biopsies	12	G.F.	–	–	+	+	–
	13	M.P.	–	–	+	–	–
	14	S.B.	–	–	+	+	–
	15	M.E.	–	–	+C	+C	–
	16	D.M.	–	–	+	+	–
	17	S.P.	–	+	+	+	–
			0	1	9	8	0

Cont'd .....

Cont'd:

Treatment		Name	IgG	IgM	IgA	C_3	C_1q
No treatment	18	B.P.	-	-	+	+	-
	19	M.H.	-	-	+	-	-
Uninvolved	20	A.T.	-	-	+	-	-
	21	G.F.	-	-	+	-	-
9 Biopsies	22	M.P.	-	-	+	-	-
	23	S.B.	-	-	+	+	-
	24	M.C.	-	-	+C	-	-
	25	D.M.	-	-	+	-	-
	26	S.P.	-	+	+	+	-
			0	1	9	3	0
G.F.D. alone	27	R.S.	-	+	+	+	+
	28	R.S.	-	+	+	-	-
8 biopsies	29	M.H.	-	+	+	+	-
	30	M.H.	-	-	+	-	-
	31	E.P.	-	+	+	-	-
	32	J.G.	+C	+	+	+	-
	33	M.R.	+	-	+	-	-
	34	J.L.	-	-	+	-	-
			2	5	8	3	1
34 Biopsies			3	9	34	16	3

C = Continuous pattern of immunofluorescense

G.F.D. = Gluten-free diet.

immunoglobulin was added as the second layer.

In all 4 biopsies examined, there was no evidence of IgA secretory-piece.

Direct Immunofluorescent Studies on Skin from Patients with Adult Coeliac Disease.

21 biopsies from the uninvolved skin of patients with adult coeliac disease were examined for the presence of IgG, IgM and IgA deposits. Only 1 showed immunoglobulin deposits - as a continuous band of IgM class. No IgG or IgA papillary or continuous deposits were seen.

Normal Skin from Volunteers.

9 biopsies from five healthy volunteers and 4 in-patients with cardiac ischaemia or chronic bronchitis, who consented to skin biopsy were examined for IgG, IgM and IgA deposits. No continuous or papillary deposits of immunoglobulins as seen in D.H. skin, were observed.

'GLobules/Globular Deposits'.

These are shown in Figure IV. 29. In the initial pilot studies on the skin of patients with D.H. and coeliac disease these well defined specifically staining immunofluorescent deposits were seen. They were intensely bright and appeared 'globular' and homogenous. The term 'globules' was therefore used to describe them. They were found in 6 of the 18 skin biopsies from D.H. patients and in 14 of 21 patients with adult coeliac disease. However, they were also found in a high proportion (in 6) of 8 skin biopsies from control and healthy volunteers.

The globules were mainly of IgM or IgA class (Table IV. 14).

Group	No. of Skin Biopsies.	IgG	IgM	IgA
Dermatitis Herpetiformis	18	-	5	4
Adult Coeliac Disease	21	-	11	11
Controls	8	-	6	2

Table IV. 14. Immunofluorescent 'Globules' in Skin of Patients with Dermatitis Herpetiformis, Adult Coeliac Disease and Controls.

The 'globules' were found in the upper dermis, at the dermo-epidermal junction, or in the basal layers of the epidermis itself. The situation and structure of these globules could not be correlated in any way to any known histological entity and their significance is at present unknown. It would appear that they are not due to non-specific uptake of fluoroscein, for they were found to be mainly associated with the IgM or IgA class FITC conjugates and very rarely with IgG (further studies indicate that IgG 'globules' are also existent - though they were not found in this initial pilot study). Specificity tests and blocking experiments however, have not been performed to elucidate further the immunological specificity of these 'globules'.

The incidence of these 'globules' was equally high in both test and control biopsies (Table IV. 14) and indicate that they may be a feature of normal skin and not specific for D.H. or coeliac disease skin. For this reason, further assessment, and quantitation of these deposits in later skin biopsies were not persued.

Immunofluorescent Studies of the Skin in 'non-D.H.' and Various other Skin Disease Patients:

54 skin biopsies from 48 patients with various skin disorders were examined by direct immunofluorescence. All 54 were examined for IgG, IgM and IgA and some for the C3 and/or C1q components of human complement. For the C3 and C1q, direct and indirect immunofluorescence were used.

The 48 patients comprised:

Cutaneous vasculitis 10
Erythema multiforme 1
Oral erosions 2
Bullous pemphigoid 3
Pemphigus 1
Discoid L.E. 1
Erythema nodosum 1
Dapsone responsive rash - thought not to be D.H. 15
Eczema 1
Erythema induratum 2
Scleroderma 1

Miscellaneous Skin Disorders 7

Blistering eruption ? cause 4

Irritant rash ? cause 4

? L.E. 1

Glomerulonephritis - (no skin lesions) 3

The results of the immunofluorescent studies on these patients are tabulated in Table IV. 15. It is of note that in all 54 biopsies, IgA was never detected in either the involved or uninvolved skin. IgG was found in 6 patients (one pemphigus, in the epidermal intercellular cement substance; one bullous pemphigoid along the basement membrane and in 4 patients in the dermal blood vessels - two had erythema induratum, one vasculitis, and one patient had glomerulonephritis with no skin lesions). All these four latter patients also had IgM and C3 in the same situations.

IgM was found on its own in one further patient with bullous pemphigoid (alone the basement membrane).

In one patient, C3 and C1q in the absence of IgG, IgM and IgA, were found scattered in the dermis. This patient (Price) has severe cutaneous vasculitis with evidence of internal involvement (proteinuria, epistaxis and intestinal bleeding). She died subsequently and post-mortem examination indicated that she had polyartereitis nodosa. She also had circulating immune complexes, very low haemolytic serum complement and no C1q was detected in her serum.

Histocompatibility Antigens.

The results of the histocompatibility typing for HL-A and 4a and 4b complex of antigens in the 39 D.H. and 36 ACD patients are represented schematically in Tables IV. 16 and 17).

Significantly raised incidences (when each antigen was evaluated independently of the others) were found with the HL-A 1, HL-A 8, HL-A 1 and 8 together, and 4b antigens in both D.H. and ACD groups and these are shown in Table IV. 18. However, as some of these antigens are linked to each other genetically, the true significance of these raised incidences has to be assessed with this in mind, and this will be done later.

Table IV. 15 : Immunofluorescent Studies in non-D.H. Patients and in Patients with Various other Skin Diseases

No.	Patient	Skin Biopsy	IgG	IgM	IgA	C3	C1q	Diagnosis
1.	Syms	Uninvol.	-	-	-	ND	ND	Erythema Multiforme
2.	O'Connell	Invol.	-	-	-	-	-	Cryoglobulinaemic vasculitis
3.	Toplar	Uninvol.	-	-	-	-	-	Vasculitis
4.	* Da Costa	Uninvol.	-	-	-	-	-	Oral erosions
5.	* Da Costa	Invol.(Gum)	-	-	-	-	-	Oral erosions
6.	Tippen	Uninvol.	-	+ BM	-	-	-	Bullous Pemphigoid
7.	*Graham	Invol.	-	-	-	-	ND	Vasculitis
8.	*Graham	Uninvol.	-	-	-	-	ND	Vasculitis
9.	O'Sullivan	Uninvol.	-	-	-	-	-	Vasculitis
10.	*Tweed	Invol.	-	-	-	-	-	Vasculitis
11.	*Tweed	Uninvol.	-	-	-	-	-	Vasculitis
12.	*Gonzales	Invol.	-	-	-	-	ND	Erythema Nodosum
13.	*Gonzales	Uninvol.	-	-	-	-	ND	Erythema Nodosum

Cont'd .....

No.	Patient.	Skin Biopsy	IgG	IgM	IgA	C3	C1q	Diagnosis
14.	Angol	Invol.	-	-	-	-	ND	Blistering eruption ? cause
15.	Weener	Uninvol.	-	-	-	-	-	Glomerulonephritis
16.	Olive	Invol.	+I/C	-	-	ND	ND	Pemphigus
17.	Howard S.	Uninvol.	-	-	-	ND	ND	Dapsone responsive rash - not D.H.
18.	Tuvey	Uninvol.	-	-	-	ND	ND	- ditto -
19.	Melville	Invol.	-	-	-	-	ND	Blistering eruption ? cause
20.	Howard D.	Uninvol.	-	-	-	ND	ND	Dapsone responsive rash - not D.H.
21.	Soanes	Uninvol.	-	-	-	ND	ND	- ditto -
22.	Stalley	Uninvol.	-	-	-	ND	ND	- ditto -
23.	Francis	Uninvol.	-	-	-	ND	ND	- ditto -
24.	Carson	Invol.	-	-	-	ND	ND	Blistering eruption ? cause
25.	White	Invol.	+ BV	+ BV	-	+ BV	-	Vasculitis
26.	Venables	Uninvol.	+ BV	+ BV	-	+ BV	-	Glomerulonephritis
27.	Margolis	Uninvol.	-	-	-	-	-	Blistering eruption ? cause

Cont'd .....

No.	Patient	Skin Biopsy	IgG	IgM	IgA	C3	C1q	Diagnosis.
28.	Wisdale	Invol.	+ BV	+ BV	-	+ BV	-	Erythema induratum
29.	Lunn	Invol.	-	-	-	-	-	Glomerulonephritis
30.	Sinclair	Uninvol.	-	-	-	-	ND	Dapsone responsive rash - not D.H.
31.	Burrows	Uninvol.	-	-	-	-	ND	- ditto -
32.	Bedford	Uninvol.	-	-	-	-	-	Vasculitis
33.	Goldstein	Uninvol.	-	-	-	-	ND	Dapsone responsive rash - not D.H.
34.	Lucas	Uninvol.	-	-	-	-	ND	- ditto -
35.	Milliken	Uninvol.	-	-	-	-	ND	- ditto -
36.	Milliken	Uninvol.	-	-	-	-	ND	- ditto -
37.	Snaillum	Uninvol.	-	-	-	-	ND	- ditto -
38.	Kennan	Invol.	+ BM	-	-	ND	ND	Bullous Pemphigoid
39.	Skipworth	Uninvol.	-	-	-	ND	ND	Eczema
40.	Minns	Uninvol.	-	-	-	ND	ND	Mouth ulcers
41.	Price	Invol.	-	-	-	+?BV	+?BV	Vasculitis
42.	Cauzmar	Invol.	-	-	-	-	-	Bullous pemphigoid

Cont'd .....

No.	Patient	Skin Biopsy	IgG	IgM	IgA	C3	Clq	Diagnosis
43.	Cheung	Invol.	-	-	-	-	-	Irritant rash ? cause
44.	Larkin	Invol.	-	-	-	ND	ND	? L.E.
45.	Porter	Invol.	-	-	-	ND	ND	Scleroderma
46.	Cook	Invol.	-	-	-	ND	ND	Dapsone responsive rash - not D.H.
47.	Hudson	Uninvol.	-	-	-	ND	ND	- ditto -
48.	*Robertson	Uninvol.	-	-	-	ND	ND	- ditto -
49	*Robertson	Uninvol.	-	-	-	ND	ND	- ditto -
50.	Spencer	Invol.	-	-	-	ND	ND	Vasculitis
51.	Ferry	Invol.	-	-	-	ND	ND	Discoid L.E.
52.	Tregiden	Invol.	-	-	-	ND	ND	Vasculitis
53.	Strenger	Uninvol.	-	-	-	-	ND	Irritant rash ? cause
54.	Poole	Invol.	+BV	+BV	-	+BV	-	Erythema induratum

ND = Not done
BM = Basement membrane
I/C = intercellular

BV = Blood vessels.
* = Repeat of same patient

In the 100 controls, HL-A 1 was found in 32%, HL-A 8 in 26% and HL-A 1 and 8 together in 19%. Of the 923 controls typed for the 4a, 4b antigen, 786 (85%) typed 4b positive, 337 (37%) had 4b alone, whilst 449 (49%) typed, 4a, 4b positive. In 137 (15%) 4a was found alone.

In the patients, HL-A 1 was present in 23 (58%) of the 39 D.H. patients, and in 27 (75%) of the ACD patients. HL-A 8 was present in 30 (77%) of the D.H. patients and 32 (88%) of the ACD patients. HL-A 1 and 8 together were present in 22 (56%) of the 39 D.H. patients and in 27 (75%) of the 36 ACD patients.

The incidence of HL-A 1 was significantly greater in both the D.H. ($p < 0.01$) and ACD ($p < 0.001$) groups as were the incidences of HL-A 8 alone ($p < 0.001$) and HL-A 1 and 8 together ($p < 0.001$). The deficit of other antigens in the patients, accompanying the increase in HL-A 1 and HL-A 8 is widely distributed and there is no significant deficit in the frequency of any particular antigen.

4b was found in 38 of the 39 D.H. patients. The one exception was the Egyptian Arab patient. 4b was found in all 36 ACD patients.

The incidence of the 4a antigen was not significantly different from the control population at the 5% level. The differences in the incidence of 4b is significantly greated in controls in both the D.H. and ACD groups ($p < 0.05$), and was only absent in the one non-Caucasian patient in this series.

True Significance of the Raised Incidences of HL-A 1, 8, 1 and 8 together, and 4b Antigen.

The raised incidences of these antigens in this study has to be evaluated against the background of the genetics of the HL-A system. Thus, it is known that HL-A 1 and HL-A 8 are products of closely linked gene loci and in North Western European populations there is a linkage disequilibrium resulting in the frequent inheritance of a chromosome HL-A 1, 8 (Albert et al. 1971). The increased incidence of HL-A 1 in this study is consistent with this hypothesis and is probably a result of the greater increase in HL-A 8, and not itself an independent phenomenon. This finding is in agreement with Falchuk and Strober

(1972). Similarly the 4b antigen is very highly associated with HL-A 8, and when viewed in this light, the raised incidence in this series is not significant. The only true significant finding in this series therefore, is an increased incidence of HL-A 8.

Small Intestinal Findings.

All the patients with ACD had by definition, a flat or flat-with-mosaic proximal jejunal mucosa. Of the 38 biopsies from the D.H. patients, 16 (42%) had a normal (leaves or fingers-and-leaves) macroscopic appearance, whilst 22 (58%) were abnormal (flat or convoluted).

Relation of HL-A 8 to Small Intestinal Findings in D.H.

In the D.H. group with macroscopically normal small intestinal biopsy, the incidence of HL-A was 88%. In the macroscopically abnormal group, HL-A 8 was present in 63%. There is no significant difference between these two groups.

There was also no significant difference in the incidence of HL-A 1 and HL-A 1 and 8 together between the two D.H. groups. Similarly, there was no difference between the incidence of HL-A 1 and HL-A 8 in the D.H. patients and the ACD group, but there was a slightly significant difference between the incidence of HL-A 1 and 8 together in the total D.H. group and the patients with ACD ($p < 0.05$).

Table IV : 16

Tissue Typing Results in 39 Patients with Dermatitis Herpetiformis.

	HL-A 1	HL-A 8	Others	4a	4ab	4b
1.	+	+	3, 12	-	+	-
2.	-	+	2, 12,	-	+	-
3.	-	+	2, 12, W28	-	+	-
4.	+	+	W18	-	-	+
5.	-	-	2, 5, 12, Bt15	-	+	-
6.	+	+	12, Bt15	-	+	-
7.	+	-	3, 5, W5	-	+	-
8.	-	+	2, 3, 7	-	-	+
9.	+	+	12, Bt15	-	+	-
10.	+	+	11, W22	-	-	+
11.	-	-	2, 12, Bt15, W18	-	+	-
12.	+	+	2, W5	-	-	+
13.	+	+		-	-	+
14.	+	+	2, W10	-	-	+
15.	+	+	2, W27	-	+	-
16.	-	-	2, W18, W27	-	+	-
17.	+	+	3, W10	-	-	+
18.	+	+	7	-	-	+
19.	-	-	3. 12, Ym, W22	-	+	-
20.	+	+	11, W5	-	-	+
21.	+	+	12, Ym	-	+	-
22.	+	+	3, 7	-	-	+
23.	-	-	3, 7, 9, W18	-	+	-
24.	+	+	3, 7	-	-	+
25.	-	+	2, 9, 12	-	+	-
26.	-	+	2, 9, W21	-	+	-
27.	+	+	12, Ym	-	+	-
28.	-	-	9	-	+	-
29.	+	+	2, 7	-	-	+
30.	-	+	9, W32, W18	-	-	+
31.	+	+	11, W18	-	-	+
32.	+	+	9, W5	-	-	+
33.	-	-	9, 10, 12, W18	-	+	-
34.	+	+	12, W29	-	+	-
35.	+	+	2, W15	-	-	+
36.	-	+	12	-	+	-
37.	-	+	7, 9	-	+	-
38.	-	-	5, 9, 10	+	-	-
39.	+	+	W18	-	-	+

TableIV :17

Tissue Typing Results in 36 Patients with Adult Coeliac Disease.

	HL-A 1	HL-A 8	Others	4a	4ab	4b
1.	-	+	3, 7	-	-	+
2.	+	+	2, W18	-	-	+
3.	+	+	3, W18	-	-	+
4.	+	+		-	+	-
5.	+	+	W18	-	-	+
6.	+	+	12	-	+	-
7.	-	+	2, 7	-	-	+
8.	+	+	2	-	-	+
9.	+	+	12	-	+	-
10.	-	-	10, 12, Da25	-	+	-
11.	+	+	5	-	+	-
12.	-	-	2, Da25, W17, W22	-	+	-
13.	+	+	10	-	+	-
14.	-	+	2, W10	-	-	+
15.	+	+	3, W15	-	+	-
16.	+	+	10, 12	-	+	-
17.	-	+	2, 9, W18	-	-	+
18.	+	+	2	-	-	+
19.	+	+	2, W27	-	+	-
20.	+	+	2, 12	-	+	-
21.	+	+	Da25	-	+	-
22.	+	+	2	-	+	-
23.	+	+	12, W32	-	+	-
24.	+	+		-	-	+
25.	+	+	9, 12	-	+	-
26.	+	+	3, 7	-	-	+
27.	-	+	2, 3, 12	-	+	-
28.	+	+	12, W29	-	+	-
29.	-	-	Da25, W10, W18	-	-	+
30.	+	+	12	-	+	-
31.	+	+	10	-	+	-
32.	+	+	5, Da25	-	+	-
33.	+	+	2, W15	-	-	+
34.	-	-	3, 5, 7, W32	-	+	-
35.	+	+	2, 12	-	+	-
36.	+	+	12, W29	-	+	-

TABLE IV.18

	Controls (total 100)	D.H. (total 39)	A.C.D. (total 36)
HL-A 1	32 (32%)	23 (58%)	27 (75%)
HL-A 8	26 (26%)	30 (77%)	32 (88%)
HL-A 1 & 8	19 (19%)	22 (56%)	27 (75%)

Incidence of HL-A 1, HL-A 8 and HL-A 1 and 8 in controls and patients with D.H. and A.C.D.

	D.H. Patients		A.C.D.
	Normal Macroscopic Appearance	Abnormal Macroscopic Appearance	
Total	16	22	36
HL-A 8 positive	14 (88%)	15 (63%)	32 (88%)
HL-A 8 negative	2 (12%)	7 (37%)	4 (12%)

Table IV. 19 - Relative incidence of HL-A 8 positivity within the D.H. and adult coeliac disease (A.C.D.) groups when compared with macroscopic appearance of small-intestinal biopsies.

Small Intestinal Biopsy Findings in D.H. Patients.

Patients. In this study, small intestinal biopsies from 43 patients (21 males and 22 females) with D.H. were studied. At the time of initial biopsy, all the patients were on a normal diet. 20 'control' biopsies were examined. These comprised 1 biopsy from a healthy volunteer physician, 11 from patients with eczema, 7 from patients with psoriasis, and 1 from a patient with pemphigus. The patients with eczema and psoriasis had small intestinal biopsies carried out to determine if there was any abnormality of the small intestine in these conditions; the patient with pemphigus had a biopsy performed as she had been diagnosed at another hospital as having D.H.

Results.

Macroscopic Appearance.

Of the 20 biopsies from the patients who did not have D.H. (controls), 3 showed leaves only and 17 fingers and leaves. Among the 43 D.H. biopsies, the mucosa was flat in 13, convoluted in 10, leaves only in 8 and fingers and leaves in 12.

Total Lymphocytic Counts. (Figure IV. 39)

In the control group, the mean lymphocyte count per 1000 epithelial cells was 160 $\pm$ S.E. 13 (range 69 - 285). Only 4 of the control subjects had counts greater than 200. In the patients with D.H. the mean lymphocyte count per 1000 epithelial cells was 464 $\pm$ S.E. 27 (range 313 - 627) for the flat biopsies; 365 $\pm$ S.E. 59 (range 130 - 739) for the convoluted biopsies; 535 $\pm$ S.E. 39 (range 408 - 727) for the leaves only biopsies; 301 $\pm$ S.E. 31 (range 121 - 445) for the fingers and leaves biopsy specimens. All four macroscopic groups showed a highly significant difference in mean count compared to the control group ($p < 0.001$). Ten of the D.H. patients had total counts less than 300, in 8 less than 285 i.e. within the control range, and 3 had counts less than 200. Of these 3 patients 2 showed fingers and leaves. The last one who had a convoluted biopsy, had a reticulum cell sarcoma of the small intestine excised

5 years previously.

The group with the highest mean lymphocyte count was the leaves only group. However, this was not significantly higher than the mean count for the flat biopsies ($p > 0.10$), but it was significantly higher than the fingers and leaves group ($p < 0.001$). The mean count for the fingers and leaves group was significantly lower than that for the flat biopsies ($p < 0.001$), but not significantly different from that of the convoluted specimens ($p > 0.40$).

Position of the Intraepithelial Lymphocytes.

Basal Position. (Figure IV. 40). The mean number of lymphocytes situated in the basal position was 79 ± S.E. 8 (range 28 - 159) in the control group; 89 ± S.E. 11 (range 43 - 191) in the flat biopsies; 93 ± S.E. 12 (range 46 - 177) in the convoluted biopsies; 121 ± S.E. 18 (range 43 - 205) in the leaves only biopsies; and 98 ± S.E. 13 (range 33 - 187) in the fingers and leaves biopsies. There was no significant difference in the mean number of lymphocytes situated in the basal position between the control group and any of the D.H. groups except the 'leaves' only group ($p < 0.02$). There was no significant difference between the four D.H. groups with regard to their mean basal lymphocyte counts. The four control subjects whose total counts were greater than 200 had basal cells of 87, 127, 143 and 159 which, in 3, formed more than 50% of the total lymphocytes, while in none of the 7 D.H. patients with lymphocyte counts between 200 and 300, did basal cells form more than 50% of the total count (Table IV. 20).

Among the 4 control subjects the mean ratio of basal to total counts was 49% (1 : 2), and among the 7 D.H. patients this ratio was 23% (1 : 4).

Perinuclear Position. (Figure IV. 41). The mean number of lymphocytes in the perinuclear position per 1000 epithelial cells was 81 ± S.E. 7 (range 40 - 145) in the control group; 362 ± S.E. 23 (range 252 - 532) in the flat biopsies; 251 ± S.E. 46 (range 80 - 421) in the convoluted biopsies; 402 ± S.E. 43 (range 225 - 510) in the leaves only biopsies; and 195 ± S.E. 25 (range 34 - 324) in the fingers and leaves biopsies. Only 5 of the D.H. patients had perinuclear

counts in the control range (less than 145). Compared to the control group, the mean perinuclear lymphocyte count was significantly raised in each of the four D.H. groups ($p < 0.001$). The highest mean count for the D.H. groups was in the leaves only specimens. There was however, no significant difference between the mean perinuclear count in the flat biopsies and the leaves only group ($p > 0.30$). The lowest mean count was in the fingers and leaves group, but this was not significantly less than the next lowest group, the convoluted specimens ($p > 0.2$).

Supranuclear Position. (Figure IV. 42). The mean number of lymphocytes in the supranuclear position per 1000 epithelial cells was 0.55 ± S.E. 22 (range 0 - 3) for the control group; 13.7 ± S.E. 2.3 (range 3 - 26) for the flat biopsies; 20.1 ± S.E.6.9 (range 1 - 69) for the convoluted biopsies; 14.5 ± S.E. 3.3 (range 4 - 29) for the leaves only biopsies; 8.3 ± S.E. 2.6 (range 0 - 25) for the fingers and leaves biopsies. Eight of the D.H. patients showed supranuclear counts of 3 or less, i.e., within the control range.

The mean supranuclear lymphocyte counts were significantly greater in all four D.H. groups compared to the control ($p < 0.001$). There was no significant difference between any of the four D.H. groups.

Ratio of Perinuclear to Basal Lymphocyte Count as an Index of Intestinal Abnormality.

It has been noted that, except for the 'leaves' only group ($p < 0.02$), there was no significant difference between the basal counts of the controls and D.H. groups. The main increase was found in the perinuclear counts. As a further indicator of intestinal abnormality, the usefulness of the ratio of perinuclear to basal lymphocyte counts was examined. The results are shown in Figure IV. 43.

The mean ratio for the controls was 1.02 (range 0.42 - 2.28); for the D.H. groups - flat biopsies, mean 4.08 (range 1.95 - 8.81); convoluted biopsies, mean 2.70 (range 1.00 - 5.46); leaves only biopsies, mean 3.32 (range 1.17 - 8.37); fingers and leaves group, mean 2.00 (range 0.74 - 6.03).

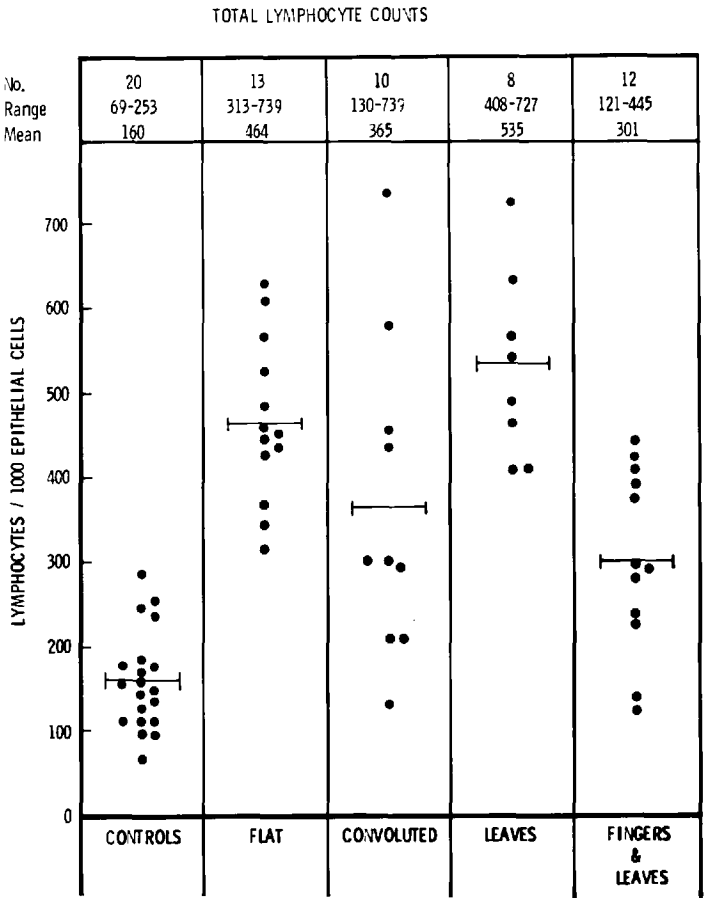


Figure IV.39 : Total intraepithelial lymphocyte counts in control and D.H. subjects.

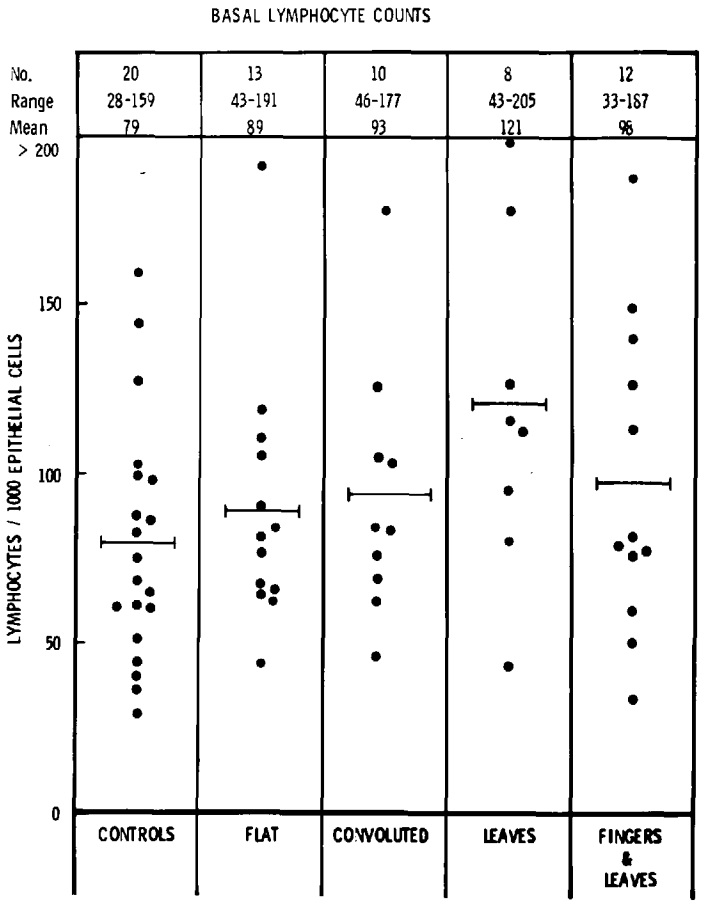


Figure IV.40 : Basal lymphocyte counts in control and D.H. patients.

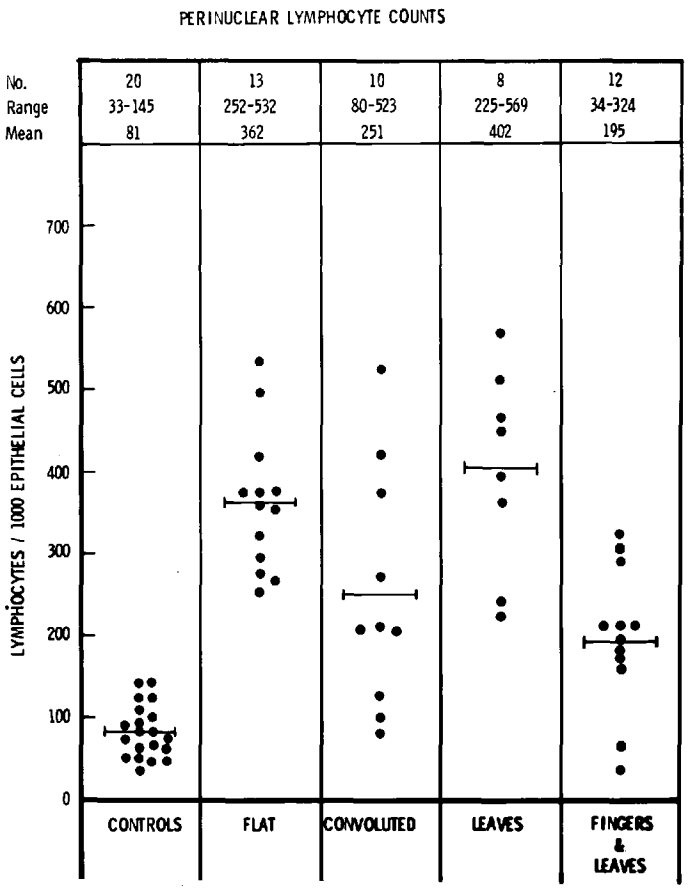


Figure IV. 41 : Perinuclear lymphocyte counts in control and D.H. subjects.

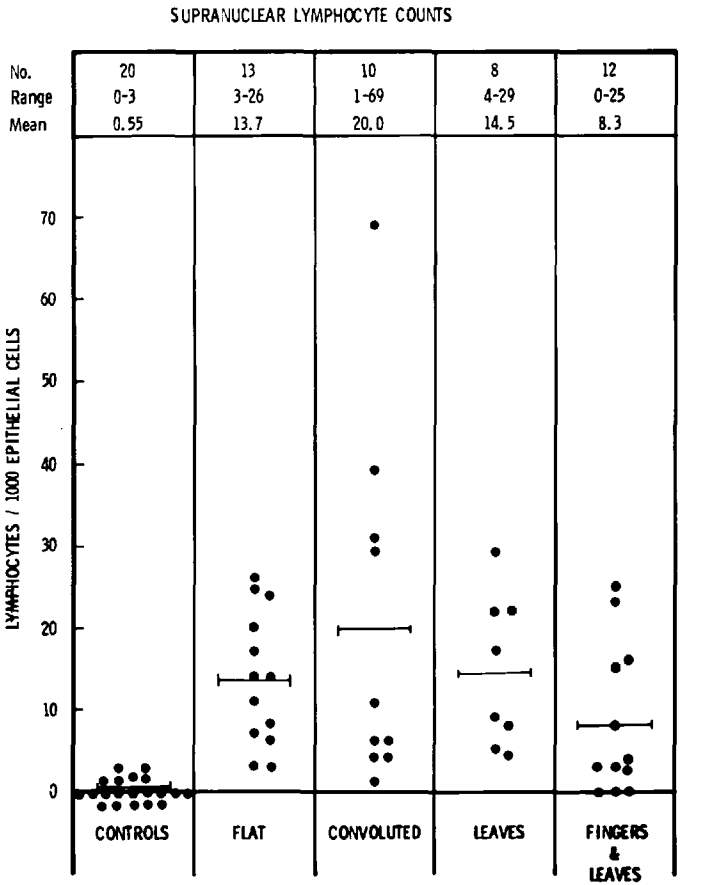


Figure IV.42 : Supranuclear lymphocyte counts in control and D.H. subjects.

Control biopsies with total counts above 200 per 1000 epithelial cells.

No	Basal	Total	Ratio	
			Basal	Total
1	142	285	1 :	2
2	159	247	1 :	1.5
3	127	253	1 :	2
4	87	235	1 :	2.5

D.H. biopsies with total counts under 285 per 1000 epithelial cells

No	Basal	Total	Ratio	
			Basal	Total
1	51	221	1 :	4
2	33	235	1 :	7
3	78	136	1 :	2
4	78	121	1 :	1.5
5	103	204	1 :	2
6	76	209	1 :	3
7	46	130	1 :	3

Table IV. 20

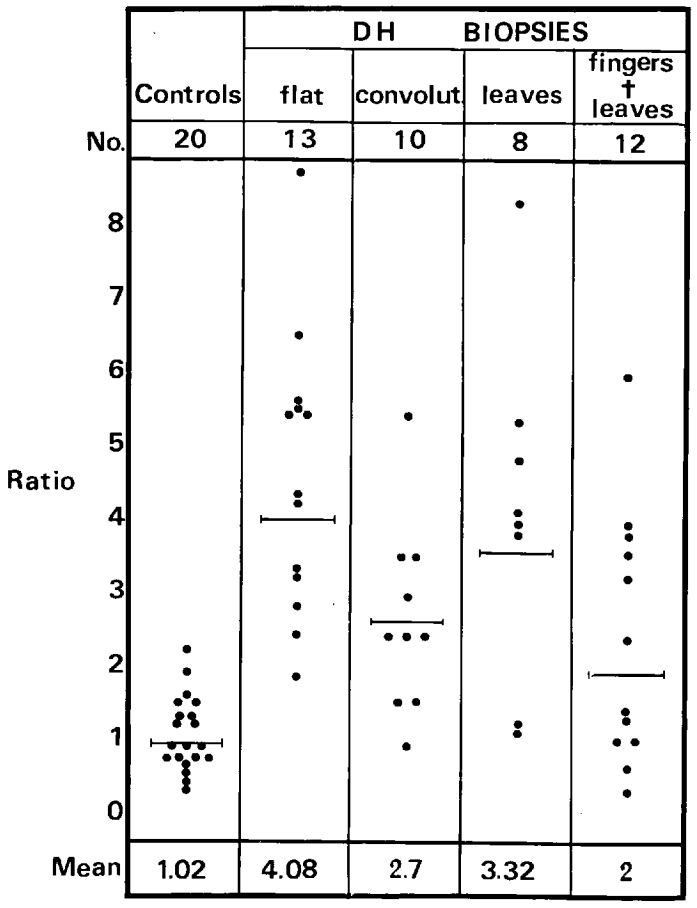


Figure IV.43 : Ratio of perinuclear to basal intraepithelial lymphocytes in 20 control subjects and 43 D.H. patients who have been divided into four categories according to the macroscopic appearances of their small intestinal biopsies.

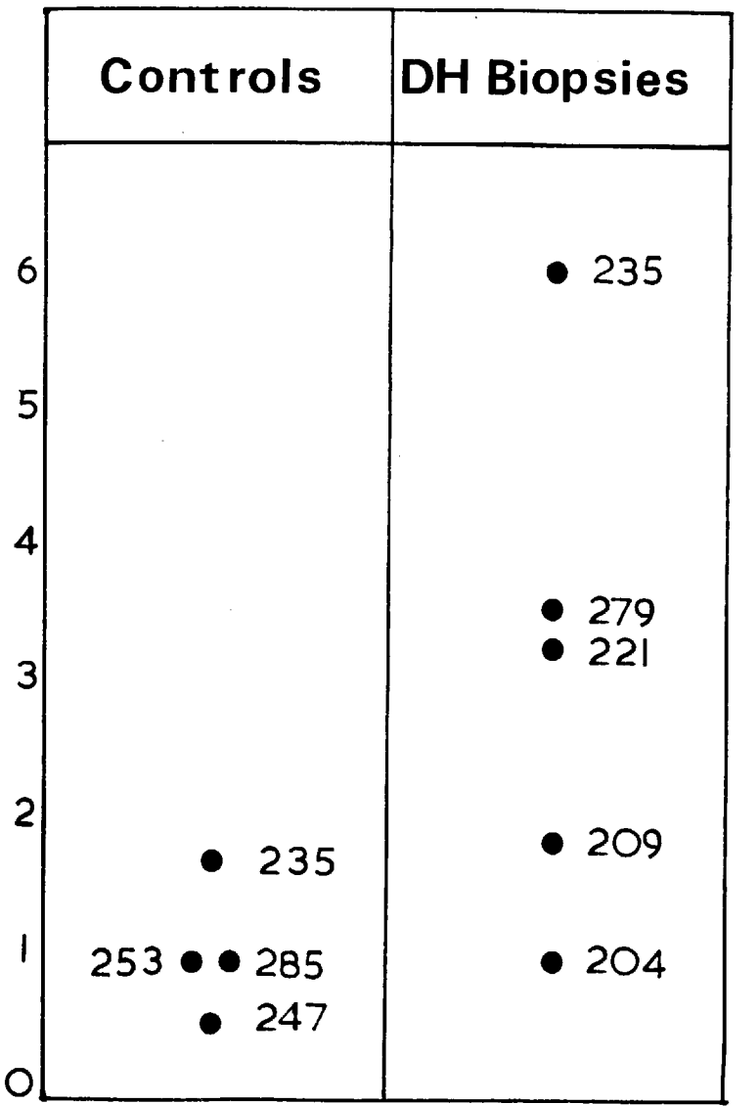


Figure IV. 44

Total intrepithelial lymphocyte counts in borderline control and D.H. patients' small intestinal biopsies (ie: >200 and <285) and relation to the ratio of perinuclear to basal lymphocyte count. In the controls, the ratio is about 1:1 whereas in the D.H. biopsies the ratio is increased despite borderline <u>total</u> lymphocyte counts.

In the 4 borderline control biopsies (total lymphocyte counts of 235, 247, 253 and 285 lymphocytes per 1000 epithelial cells), the ratio was about 1. In the 5 borderline D.H. specimens however, (Figure IV. 44), the ratio was generally higher, indicating that in this latter group, despite borderline total lymphocyte counts, the increased ratio of perinuclear to basal cells seen in definitely abnormal D.H. biopsies is also preserved.

Evaluation of the Constancy of the Macroscopic Appearance and Lymphocytic Infiltration of the Small Bowel in D.H.

In five patients who were not treated with a GFD, repeat small intestinal biopsies were performed at intervals varying between 6 months and 1 year. 4 patients had 1 repeat biopsy, whilst the fifth patient had 2. Biopsies were taken from either the fourth part of the duodenum or the duodeno-jejunal flexure. Dissecting microscope appearances of the biopsies were noted and correlated with the total and compartmental intraepithelial lymphocyte counts. The results are tabulated in Figure IV. 45.

Macroscopic Appearances. It can be seen that there is variation in the macroscopic appearances: Only 1 patient had a similar appearance in the repeat biopsy.

Intraepithelial Lymphocytes. The total intraepithelial lymphocyte count remained remarkably similar in the repeat biopsies. In addition, there was remarkably little fluctuation in the compartmental distribution of the intraepithelial lymphocytes. Similarly, the ratio of perinuclear to basal lymphocytes remained fairly constant.

Effect of a Gluten-Free Diet (GFD) on Intraepithelial Lymphocyte Count in D.H.

In 13 patients treated with a GFD for periods varying from 1 to 4 years, further small intestinal biopsies were performed. The results are shown in Figure IV. 46.

Of these 13, 7 had initially abnormal macroscopic morphology. In all 7, there was a drop in the total intraepithelial lymphocyte count on treatment with

Patient	Biopsy No.	Macro.	Lymphocyte Count				
			B	PN	S	Total	Ratio
A: A.D.	1	L	136	569	22	727	4.18
	2	L	134	518	4	656	3.86
B: G.E.	1	Flat	64	360	26	450	5.62
	2	Con.	79	374	24	477	4.73
C: Poore	1	L	80	393	17	490	4.91
	2	Con+L	57	302	6	365	5.29
D: Teal	1	L	116	468	4	588	4.03
	2	Flat	74	412	4	490	5.56
E: Burrows	1	L	95	510	29	634	5.36
	2	L	57	443	6	506	7.77
	3	Con+L	110	514	3	627	4.67

Figure IV. 45

Macroscopic appearances and intraepithelial lymphocyte counts in repeat biopsies in 5 D.H. patients on normal diet.

a GFD. The remaining 6 biopsies showed normal morphology. In 5 of these 6, the total lymphocyte count dropped on treatment with a GFD. Both these counts however, were outside the abnormal range.

The fall in the total lymphocyte count following treatment with a GFD however, seldom returns into a range which could be considered to be normal. Hence, of the 12 biopsies with raised counts, only 4 returned to a figure below 200 following treatment with a GFD.

Effect of GFD on the Distribution of Intraepithelial Lymphocytes in Relation to the Epithelial Cell Nuclei.

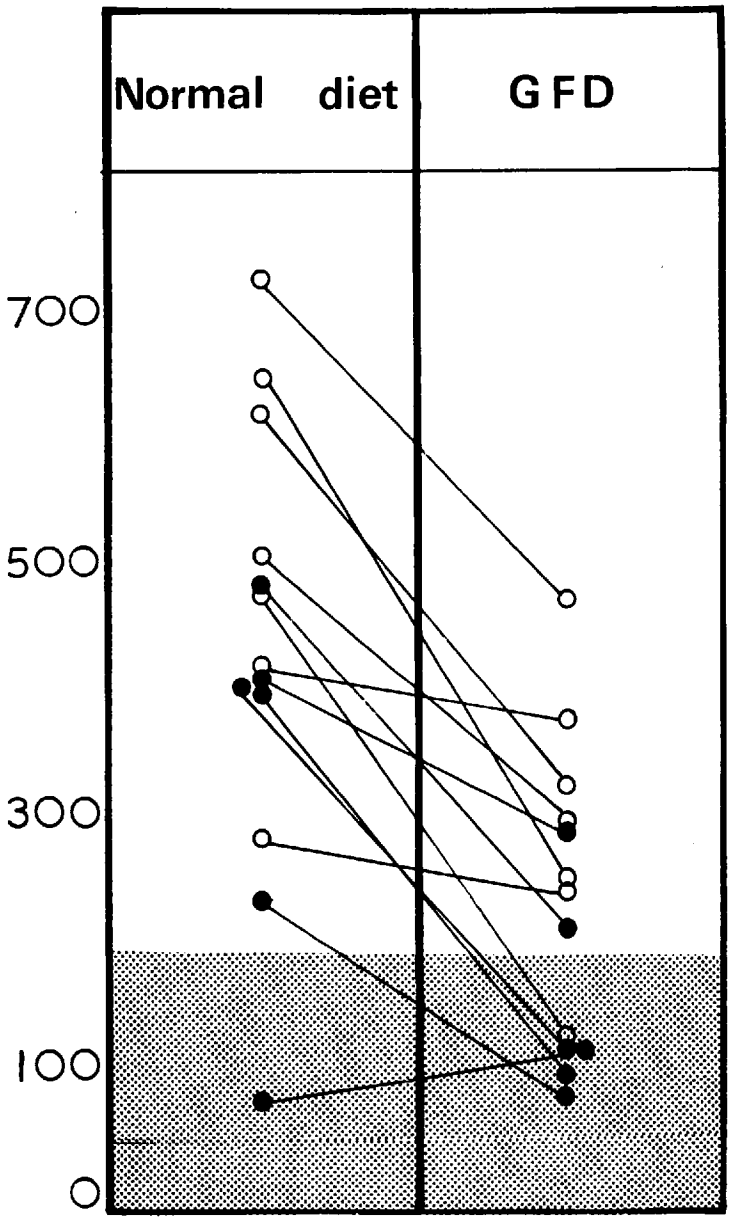
The distribution of intraepithelial lymphocytes into the basal, perinuclear and supranuclear compartments of the intestinal epithelial cells following treatment with a GFD was assessed in the same 13 D.H. patients. The results are tabulated in Table IV. 21.

It can be seen that, with a few exceptions, there is a fall in all three compartments following treatment with a GFD. The ratio of perinuclear to basal cells also falls in the majority - only 2 showing a rise - on treatment with a GFD.

Effect of Re-introduction of Gluten on the Intraepithelial Lymphocyte Count.

In 4 patients, the effect of re-introduction of gluten into the diet was assessed. Following the initial biopsy, the patients were put onto a GFD for a period of about 12 months. They then had a repeat biopsy at this time, following which they returned to a normal gluten containing diet for about three months. They were then put back onto a GFD for up to three years and then rebiopsied. The results of this study are tabulated in Figure IV. 47.

In one patient, there was a marked rise in the total lymphocyte count on gluten re-introduction. In one there was a minimal rise, whilst in the remaining two there was a fall despite re-introduction of gluten. All four patients showed a fall in the counts after prolonged withdrawal of gluten.



Patient	Before GFD		After GFD	
	Macro.	Lymph. ct.	Macro.	Lymph. ct.
1. S.I.	F	627	L	318
2. T.P.	Con.	635	L	277
3. A.T.	Con.	739	Con.	485
4. J.L	F	482	Con.	132
5. M.R.	F	429	L	389
6. G.J.	F	521	F+L	203
7. D. H.H.	Con.	295	Con.	251
8. E.L.	F+L	409	F+L	126
9. F.W.	F+L	89	F+L	136
10.T.P.	F+L	235	F+L	117
11.M.H.	F+L	374	L	304
12.R.S.	L	408	F+L	103
13.E.P.	L	490	F+L	222

open circles = flat or convoluted specimens initially
closed circle = leaves or fingers and leaves specimens initially

Figure IV.46 Total intraepithelial lymphocytes per 1000 epithelial cells in 13 patients with D.H.: Effect of a gluten free diet (GFD) of periods varying from 1 to 4 years on intraepithelial lymphocyte counts.

F = Flat; Con. = Convoluted; L = Leaves; F+L = fingers and leaves

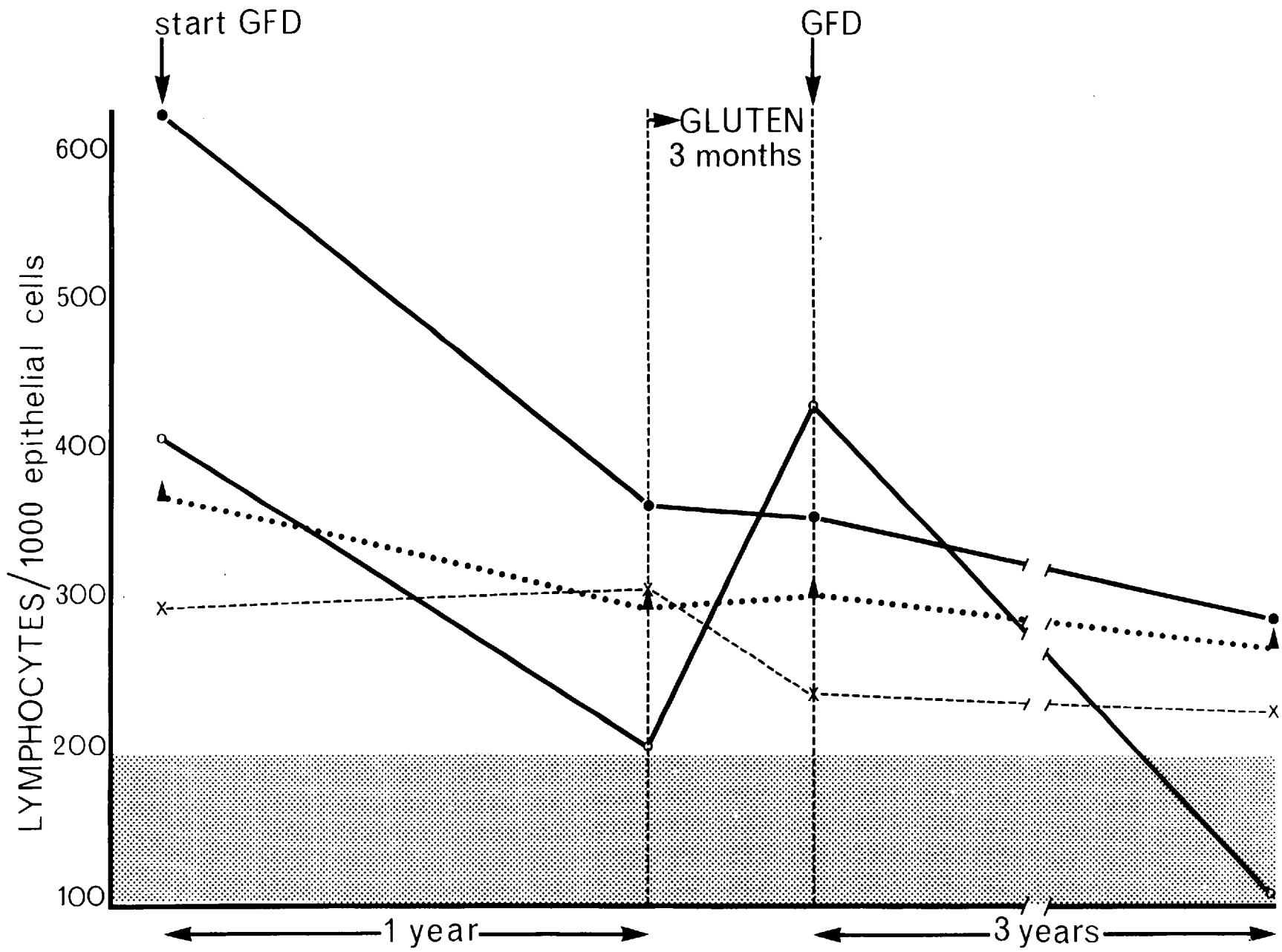
Only one however, returned into the normal range. No constant pattern of changes in the compartmental proportions of intraepithelial lymphocytes was seen with gluten challenge - but the numbers studied here are too small to draw definite conclusions.

Table IV. 21 Lymphocyte counts of D.H. small intestinal biopsies and effect of Gluten-Free Diet

	Patient		PN : B ratio	Lymphocyte Counts B	PN	SN	Total
1.	S.I.	N.D.	6.56	81	532	14	627
		G.F.D.	U	U	U	U	318
2.	T.P.	N.D.	2.50	112	383	140	635
		G.F.D.	3.10	58	180	39	277
3.	A.T.	N.D.	2.95	177	523	39	739
		G.F.D.	5.03	79	398	8	485
4.	J.L.	N.D.	3.39	106	360	6	482
		G.F.D.	1.64	50	82	0	132
5.	M.R.	N.D.	2.48	119	296	14	429
		G.F.D.	3.47	86	299	4	389
6.	G.J.	N.D.	5.52	76	420	25	521
		G.F.D.	1.23	91	112	0	203
7.	D. H-H	N.D.	3.29	62	204	29	295
		G.F.D.	2.37	74	176	1	251
8.	E.L.	N.D.	4.00	81	324	4	409
		G.F.D.	0.90	66	60	0	126
9.	F.W.	N.D.	1.63	33	54	2	89
		G.F.D.	0.74	78	58	0	136
10.	T.P.	N.D.	6.03	33	199	3	235
		G.F.D.	0.53	76	41	0	117
11.	M.H.	N.D.	3.81	76	290	8	374
		G.F.D.	2.41	89	215	0	304
12.	R.S.	N.D.	8.37	43	360	5	408
		G.F.D.	1.23	46	57	0	103
13.	E.P.	N.D.	4.91	80	393	7	490
		G.F.D.	0.77	124	96	2	222

N.D. = normal diet.
G.F.D. = gluten-free diet.

U. = undetermined.
B. = basal count
P.N. = perinuclear count.
S. = supranuclear count.



INTRAEPITHELIAL LYMPHOCYTES

Patient	Biopsy	Macro.	B.	P.N.	S.N.	Total	Ratio PN:B
1. D.H-H.	A	Con.	62	204	29	295	3.29
	B	L	59	245	4	308	4.15
	C	Flat	39	193	6	238	4.94
	D	Con.	74	176	1	251	2.37
2. T.P.	A	Con.	112	383	140	635	3.41
	B	L	132	205	38	375	1.55
	C	Con.	135	176	58	369	1.30
	D	L	58	180	39	277	3.10
3. M.H.	A	F+L	76	290	8	374	3.81
	B	F+L	89	215	0	304	2.41
	C	L	30	275	2	307	9.16
	D	F+L	67	224	2	293	3.41
4.R.S.	A	L	43	360	5	408	8.37
	B	L	60	146	3	209	2.43
	C	L	34	392	12	438	11.52
	D	F+L	46	57	0	103	1.23

Macro. = macroscopic appearance
B = Basal count
P.N. = Peri-nuclear count
S.N. = Supra-nuclear count
GFD = gluten rfee diet

Figure IV.47 : Effect of gluten withdrawal and reintroduction of gluten on the macroscopic appearance and intraepithelial lymphocyte count in the small bowel of 4 patients with dermatitis herpetiformis.

A = pre-GFD biopsy; B = After GFD for 1 year; C = Normal diet for 3 months
D = GFD for 3 subsequent years.

PART V:

DISCUSSION

INTRODUCTION

The work presented in this thesis was initially prompted by previous reports of minor immunological abnormalities in dermatitis herpetiformis (D.H.). These included the reports of serum IgM abnormalities (Fry et al. 1967, Fraser et al. 1971), splenic atrophy (Fry et al. 1967) and of immunoglobulins in the skin (Cormane 1967, van der Meer 1969). The results reported here indicate that immunological abnormalities are indeed present in D.H. and suggest that immunological mechanisms may be operative in the pathogenesis of this disease. Arising from this however, is the question as to whether these abnormalities are of primary pathogenic significance or whether they represent secondary phenomena. The answer to this is an elusive one requiring further study and cannot at the present time be answered.

The discovery of a small-intestinal abnormality in D.H. in 1966 by Marks and her colleagues and the subsequent demonstration that this is due to gluten-sensitivity, as found in coeliac disease (Fry et al. 1967, Weinstein et al. 1971) makes D.H. a unique and fascinating disease. An effort has been made therefore in this thesis, to perform parallel studies in patients with coeliac disease in an attempt to clarify the relationship between these two diseases. As in D.H., immunological abnormalities have been noted in patients with coeliac disease and in many cases, these are identical or very similar.

In view of the close relationship between D.H. and coeliac disease, the morphological studies on the small bowel in D.H. have been included in this thesis. Such morphological studies as they are presented here are not evidence of immunological abnormality, though this does not exclude an immunological aetiology. However, they have been included as supportive evidence of a much closer link between the dermatological and gastrointestinal aspects of D.H. than was previously thought, and when viewed in this light the findings discussed here may have a different significance. The increased lymphocytic infiltration found in the small

bowel in D.H. is used essentially as a diagnostic aid in this thesis. However, there is now accumulating evidence to suggest that the lymphocyte may have an immunological and pathological role in the pathogenesis of the mucosal changes seen in gluten-sensitive enteropathy (Ferguson 1974).

For the purpose of avoiding confusion and maintaining clarity, the discussion in this thesis will be confined, in its initial stages, to broad categories as presented in the results section. Each project will therefore be discussed independently and an effort will be made to bring together the major points arising from them at the end in evaluating their significance in the aetiology and pathogenesis of dermatitis herpetiformis and their possible relevance in coeliac disease and gluten-sensitivity.

ANTI-RETICULIN ANTIBODY (ARA)

In the routine testing of sera from patients with D.H. and with adult coeliac disease, an antibody reacting with connective tissue fibres present in the rat 'composite block' was observed. Two main patterns of immunofluorescent staining were seen - one well defined, the other more diffuse. In addition, multiple other patterns were observed, but in a much lower incidence when compared with the first two.

Histochemical Aspects of ARA

When compared with routine histological stains for connective tissues, the best correlation with the immunofluorescent staining was seen with the Gomori stain for reticulin. Patterns given by the histological stains for the other two main connective tissue compnents - collagen and elastin, were completely different, and the antigen against which the antibody was directed was not considered to be located in these fibres.

It was for these reasons that the antibody was labelled as "anti - reticulin antibody". It should be stressed at this juncture however, that the term "reticulin" is a purely histological one and does not represent a well-defined or biochemically analysed substance.

Reticulin was first described in 1876 by Kupffer and was entirely

a histological concept describing a particular type of fibre demonstrated in connective tissue by light microscopy. Collagen fibres are birefringent, non-branching and occur in coarse fibres which stain only lightly with silver impregnation techniques, becoming yellowish-brown in colour. Reticulin fibres, in comtrast, occur singly but branch with one another and are 'argyrophilic' - staining an intense black with silver (Maresch 1905).

Ever since its discovery, a controversy has raged over the nature of reticulin - in particular as to whether it was identical to, or distinct from collagen. Some consider that reticulin and collagen are fundermentally identical (Mallory and Parker 1927) whereas others beleive that they are different only in their physical properties (Foot 1927, Dublin 1946, Windrum et al. 1955). Others beleive that they are entirely different, both physically and chemically (Glegg et al. 1953, Puchtler1964) A major practical difficulty is that though reticulin is widely distributed it is rarely a major constituent and is consequently difficult to isolate in quantities in a pure form sufficient for chemical analysis (Glegg et al. 1953, Sulitzeanu 1967). Bowes and Kenten (1949) studied the amino acid composition of reticulin and reached the conclusion that it was similar to collagen, but that the proline and hydroxyproline values were lower. Windrum et al. (1955) in a study of reticulin isolated from the mid-cortical zone of human kidneys reached the same conclusions.

Using the electron microscope, Melcher (1966) showed that reticulin is made up of rather sparsely distributed fibrils (having a 640 Å spacing as seen with collagen) within an amorphous matrix. He advanced the hypothesis that reticulin is a site for the extracellular organization of collagen from simpler units of unknown size which are produced by the fibroblasts. Later, these fibrils become incorporated into fibres and bundles characteristic of mature collagen fibres. Thus reticulin contains collagen at a particular phase of its organization. It is also most active in young developing tissues, but persists in older ones, perhaps as a template for the reorganization and maintainance of fibre

orientation in the tissue following mild damage.

Reticulin, thus, as an entity, is too large and variable in composition to be considered as a substance. For practical purposes, it remains to this day a histological entity. For this reason, the antigens against which the circulating antibodies detected in patients with D.H. and coeliac disease are reacting cannot be defined. The nearest that one can come to a satisfactory description is to say that the antigen(s) involved are present or located within the reticulin fibres as detected by silver staining. Further support that collagen is not involved comes from the collagenase experiments performed in this thesis. Thus, prolonged treatment of rat composite block sections with collagenase did not remove their ability to react with ARA positive sera. Parallel studies with van Giesen stains showed complete removal of collagen. With the Gomori stain for reticulin however, there was no significant change after treatment with collagenase.

With the horse-radish peroxidase method, staining patterns very similar to those seen using immunofluorescence were obtained with ARA positive sera.(Figures IV. 14 - 17) Again, the pattern of staining was similar to that seen with the Gomori stain for reticulin. The immuno-electron microscope studies interestingly show that the antigens reactive with ARA are closely related to the collagen fibres in the connective tissue bundles (Figures IV. 18 and 19). The collagen fibres themselves however, were not involved (Figure IV.20) and it would appear that the very closely associated surrounding matrix is the main site involved. This finding is consistent with the idea of Melcher (1966) previously mentioned that reticulin is a site for the extracellular organization of collagen fibrils from simpler units produced by the fibroblasts.

Further support for the close relationship of ARA with connective tissue comes from the studies using connective tissue antisera, and the accompanying absorption experiments. With anti-bovine heart valves (BHV) immunofluorescent patterns very similar to those obtained with ARA were seen. The close association of the antigens involved is

supported by the absorption studies where total removal of ARA activity in human sera was effected. Similar results were obtained with the non-collagenous reticulin component (NCRC) of pig and human tissues. In fact with these latter antisera, a much closer identification of the reticulin pattern given by ARA was obtained and it is probable that the antigen(s) responsible for ARA are contained in the NCRC chemically characterised by Pras and Glynn (1973). These authors extracted a saline insoluble residue of porcine and human tissues (liver and kidney) by a distilled water dispersion method. They found it to be a protein distinct from collagen in that it contained no hydroxyproline and had the glycine content of normal proteins (9%). In a further communication, Pras et al. (1974), in a study of the immunofluorescent staining seen with antisera to NCRC, showed very similar patterns to those produced by ARA and silver staining. They considered NCRC to be a major component of reticulin and found it to be strongly immunogenic. In addition, they found the antibodies to porcine and human NCRC to have both species-specific and shared antigenic determinants. Staining patterns similar to those produced by ARA were seen with stromal reticulin, basement membranes, connective tissue between epithelial cells in various endocrine organs, in muscle and in kidney tubules. Interestingly, they found no staining with skin, a situation similar to that found with ARA in this thesis.

The variable immunofluorescent staining patterns seen with the ARA from patients with D.H. and coeliac disease indicate that a spectrum of antigens, rather than a single one, are involved. Such a situation is not uncommon in the study of autoantibodies as detected by immunofluorescence. Hence, with anti-nuclear antibodies, a wide spectrum of patterns are seen (Beck 1961). These include homogenous, speckled and nucleolar patterns and are attributed to the participation of various nuclear components e.g: RNA, single and double-stranded DNA, in the production of what are essentially a heterogeneous group of antibodies. A similar situation exists with smooth muscle antibodies (Holborow 1972) and with

mitochondrial antibodies (Doniach and Walker 1974).

The absorption studies with gluten and gluten fraction-3 which will be discussed more extensively later on, indicate that there may be common cross-reacting antigens in gluten and reticulin. The failure of complete removal of the ARA staining and the modification of immunofluorescent staining in some of the sera indicate that many other antigens not present in gluten are also involved in the production of ARA.

Rizzetto and Doniach (1973) in a study of the immunofluorescent patterns given by 'reticulin' antibodies in human sera considered that at least five types of antibodies reacting against distinct antigens of intra- and extracellular components in mesenchyme could be identified. Two types of fibrillar antigens could be distinguished on the basis of the staining patterns , anatomical distribution and species specificity. These two patterns were the most clearly distinguished and were refered to as reticulin 1 (R1) and reticulin 2 (R2). In the R1 pattern, the main features were a nodular or lumpy fluorescence surrounding the portal tracts up to the limiting plate of hepatocytes, and a fine outline of sinusoids. Discrete, wavy worm-like strands were also seen in the higher titre sera. In the kidney, R1 sera showed peritubular and Bowman's capsule staining. These antibodies were foundto react with animal and human tissues. These patterns corresponded to the'typical' ARA patterns described in this thesis and were most commonly found in this study in patients with D.H. and with childhood and adult coeliac disease.

With the R2 pattern, Rizzetto and Doniach (1973) found staining of the thin sharp-edged fibres around the portal tracts, but not extending into the liver lobules. It was seen as streaks between gastric glands and concentrated around blood vessels in all organs. In the kidney, except for perivascular staining, there was no other significant immunofluorescence. The R2 pattern, as described by these authors, closely approximates those illustrated in figures IV.8 and 9 in this thesis. They were found in non-D.H. and non-coeliac disease patients and were considered not to be typical of the pattern associated with these disease groups.

The other three patterns described by Rizzetto and Doniach (1973) were Kupffer cell fluorescence, sinusoidal cell cytoplasmic fluorescence and a broad group - reticulin sinusoidal fluorescence patterns (Rs). The majority of patients giving reticulin connective tissue immunofluorescence on rat tissue fell into this last mixed group. The common feature was a diffuse cytoplasmic staining of Kupffer cells and other reticulo-endothelial cells. In many sera R1 and less commonly R2 patterns were also present, though some sera were completely negative on portal tracts while outlining all sinusoids clearly.

Such a miscellany was indeed seen in the studies reported in this thesis (figures IV. 5,6 and 10) but these were not considered to be 'typical' reticulin patterns. For this reason, they were not included as 'positives' in the study of the incidence of ARA in various diseases.

In the study of Magalhaes et al. (1974), a variety of reticulin staining patterns was also reported.

IMMUNOLOGICAL FEATURES OF ANTI-RETICULIN ANTIBODY

Immunoglobulin Class

In this thesis, ARA was found to be of IgG class in the majority of sera tested. IgM antibodies to reticulin were never found. In about 50% of the positive sera, ARA of IgA class was also found in addition to IgG. IgA antibodies on their own were not found. In the study reported by Magalhaes et al. (1974) however, IgA ARA on their own were found and this was the main immunoglobulin class in the sera of patients not on a gluten-free diet. Following gluten withdrawal however, the incidence of IgA antibodies was strikingly reduced.

In the study of Brown et al. (1973) ARA was detected in 32 (67%) of 48 children with coeliac disease. The antibody was solely of IgG class in 66% and the remaining 34% comprised a mixture of IgG and IgA. In both the studies of Brown et al. (1973) and Magalhaes et al. (1974) IgM antibodies were not found, as was the case in this thesis.

The IgA nature of a proportion of the ARA is an interesting

observation as this indicates that they are probably gut derived. IgA is the main immunoglobulin involved in the gastrointestinal tract (Chodirker and Tomasi 1963) and most gut mediated responses are IgA responses e.g: oral polio vaccination (Berger et al. 1967). The finding of IgA ARA therefore is good evidence for a relevant link between the antibody and a gastrointestinal disorder, in this case gluten-sensitive enteropathy. The finding of IgG antibodies in many patients does not necessarily make extrapolation in this direction any less relevant for it is known that immunological responses often involve IgG, IgM and IgA responses over a period of time. Hence, with polio vaccination all three immunoglobulin classes in the sequence IgM, IgG and IgA are involved (Ogra et al. 1968, Ogra and Karzon 1969). The immunoglobulin class of the antibody is therefore dependent on the time following antigenic stimulation and in a long-standing chronic disease like gluten-sensitive enteropathy it would not be unusual to find IgG as well as IgA antibodies.

ARA and Complement Fixation

ARA was found to fix complement poorly. Thus complement fixation was found in only one of 14 positive ARA sera. All 14 sera had IgG antibodies and some IgA. If they were all of IgA class only, this inability to fix complement would not be surprising as IgA antibodies do not fix complement by the classical pathway (though there is evidence to suggest that they might do so via the alternate pathway of complement activation - Spiegelberg and Gotze 1972). The inability of the IgG ARA to fix complement suggests that they belong to the subclasses IgG2 and IgG4 which are known to be non- or poor complement activators. No formal subtyping studies however, have been performed in this thesis to substantiate this. The inability of the IgA to fix complement does not necessarily mean that they are not pathogenic. Thus, there is evidence to suggest that immunoglobulins, including IgA and IgG, in an aggregated form, can trigger the alternate pathway of complement fixation (Spiegelberg and Gotze 1972). Alternatively, it has been shown that antibodies can effect damage by co-opting or activating non-sensitized

lymphocytes - "K-cells" - which subsequently attack the target organ (Perlmann et al. 1969, MacLennan et al.1969). In fact, Fakhri and Hobbs (1972) have suggested that such a mechanism could be responsible for the mucosal damage in gluten-sensitive enteropathy.

Autoantibody Nature of ARA

The autoantibody nature of ARA is indicated by its ability to react with tissue of human origin. Hence, staining of foetal skin and small bowel lamina propria was demonstrated. There was however, difficulty in staining adult human tissues and no convincing immunofluorescence was seen using many different ARA sera, with adult human skin and gut - from normal controls and coeliac disease patients. In the study of Rizzetto and Doniach (1973) however, staining was obtained with human thyroid and stomach.

The apparent discrepancy between the ability to stain foetal tissues but not adult human skin and small bowel is difficult to explain. The implications are that the antigen(s) reactive with ARA are either not present in adult human tissues or that, if they are, they are either non-reactive or concealed and not accessible to react with ARA. If one returns to the discussion on the nature of reticulin expounded earlier, the latter may in fact be a feasible explanation. Reticulin is essentially an actively turning-over tissue and therefore found mainly in immmature and young connective tissue. In the adult, it might be modified completely so as to be non-reactive or concealed from the ARA. ARA is otherwise non-species specific and, as was foundin the study of Rizzetto and Doniach (1973) reaction with tissues from a wide variety of different species was obtained.

INCIDENCE OF ARA IN D.H. AND COELIAC DISEASE

ARA was found predominantly in patients with D.H. and with childhood and adult coeliac disease. The highest incidence was found in patients with childhood coeliac disease (CCD) where the incidence was 59% of 46 patients. In adult coeliac disease (ACD) it was found in 38% of 101 patients whilst in D.H. it was detected in 22% of 50 patients. It is of note that the incidence parallels the degree of gluten-sensitivity in these three disease groups. Hence, the highest incidence was found in patients with CCD, who generally have marked gluten-sensitivity and who usually respond

very well to gluten withdrawal. At the other end, patients with mild gluten-sensitive enteropathy, as in D.H., had the lowest incidence of ARA. It is now well established that the small-intestinal lesion in D.H. is comparatively mild in the majority of patients (Fry et al. 1967, Shuster et al. 1968, Brow et al. 1971) and clinically does not give rise to signs and symptoms generally associated with coeliac disease. In fact, in the study of Fry et al. (1967) no D.H. patient had symptoms or signs suggestive of coeliac disease.

The dietary status of the patients i.e: whether they were on a gluten-free diet (GFD) or not, was very relevant. Thus, if the patients were allocated to two groups - those on a GFD and those on a normal gluten-containing diet - a very definite trend in the incidence of ARA positivity was seen. This is best illustrated in the CCD group where ARA was found in 85% of 26 patients on a normal diet, but in only 25% of 20 patients taking a GFD. With the ACD group, the incidences were 59% in those on a normal diet and only 16% in those on a GFD. The corresponding figures for D.H. were 30% and 10%.

A further example of the close relationship between ARA and the dietary status was seen in the D.H. patient M.McN. in whom serial estimations of ARA were done before and during treatment with a GFD (figure IV.22). Hence, nine months after gluten withdrawal, ARA could not be detected in her serum.

Similar incidences of ARA in D.H., ACD and CCD have been reported in various other studies. Alp and Wright (1971) found ARA in 54% of their 71 children with CCD and in 34% of 50 ACD patients. They too found an increased incidence of the antibody in patients not taking a GFD and that ARA positivity could be correlated with serum antibodies to gluten fraction-3 but not with serum antibodies to ovalbumin, or circulating immunoglobulin levels. However, each of 4 children with selective IgA defeciency and coeliac disease had strongly positive ARA.

Alp and Wright (1971) also studied the effect of re-introducing gluten into the diet of six children with treated CCD, and its effect on ARA positivity. In 4 of the 6, ARA was absent prior to gluten challenge, but

became positive after re-introduction. In a further patient, ARA was present before, and persisted after challenge. In the remaining patient, ARA was absent persistently before and after challenge.

Similar results were obtained by von Essen et al. (1972). Thus, prior to gluten withdrawal, 68% of their 40 children with coeliac disease had ARA. In some cases, the ARA fluctuated according to gluten withdrawal or re-introduction. Like Alp and Wright (1971) they could not correlate ARA with serum IgA abnormalities. Unlike them however, they found no correlation between the presence of ARA and precipitating antibody to gluten. von Essen et al. (1972) also looked at the relationship between the presence and titre of ARA and the degree of morphological abnormality in the small bowel and found no correlation.

In their study, Brown et al. (1973) found ARA in 67% of 48 patients with CCD. No attempt however, was made to relate ARA to dietary status. They too could not correlate the presence of ARA to serum immunoglobulin levels or to precipitating antibodies to gluten and other dietary antigens. Magalhaes et al. (1974) found ARA in 39% of 92 patients with ACD. When related to dietary status the marked difference in incidence noted before was again observed. Hence, ARA was found in 58% of 44 patients on a normal diet , but in only 23% of 73 patients on a GFD. In the study of Rizzetto and Doniach (1973) ARA was found in 47% of 36 D.H. patients and in 44% of 50 patients with coeliac disease.

Immunological Significance of ARA

The studies on the incidence of ARA and its relation to a GFD indicate that ARA is an antibody very closely related to gluten ingestion and gluten sensitivity. The indications are that the presence of ARA is a phenomenon directly and pathologically related to the disease and not merely a non-specific feature.

What then is this relationship? From the discussion so far, two main possibilities arise. Firstly, that gluten itself is involved, and secondly that ARA may arise as the direct result of mucosal damage following gluten ingestion. It is also possible that both these mechanisms

might contribute to the production of ARA.

Support for gluten playing a direct role comes from the absorption studies performed in this thesis using gluten and gluten fraction-3. Thus, of the 17 ARA positive sera from D.H., ACD and CCD patients, complete removal of ARA was consistently demonstrated in 3 and partial re - moval in a further 5. In all therefore, 8 - or approximately 50% - of the 17 sera were affected by the absorption procedures. Taken together, it would be reasonable tc say that these provide evidence to suggest that cross-reactivity between gluten and reticulin has been demonstrated.

When viewed more critically however, there are deficits to be explained. One is that in 9 of the 17 sera examined, there was no demonstrable absorption. Absorption studies of this sort are notoriously difficult to perform and interpret, and with this in mind, it would be of benefit to discuss at this juncture some of the points arising from the gluten absorption experiments.

A major disadvantage from the start was that one was working with ill-defined antigens. Gluten is a macromolecular complex (Ewart 1970) and knowledge of its split products in the small intestine is minimal or non-existent. In particular, precise knowledge of the toxic moiety responsible for producing the mucosal damage in gluten-sensitive entero- pathy is still not forthcoming, although it is thought to reside in the gliadin part of the complex. Reticulin, as has already been extensively discussed, is still only a histological entity and suffers much the same disadvantages as gluten in its identification as an antigen.

The failure of absorption in the 9 ARA positive sera may be due to a number of factors. It is possible that there is a failure to effect complete removal of the antibody for physico-chemical reasons. This may be due to a scacity of receptor sites, for it is well known that gluten is a largely insoluble compound. Furthermore, it is possible that the antigenic sites involved are only revealed following digestive processes in the gut. Absence of knowledge of the breakdown products of gluten makes it difficult to assess its relevance.

Failure to effect complete removal of antibody may also be attributed to the presence in the absorption mixture of soluble antigen or antigen-antibody complexes. In the latter case, if the antigen-antibody binding affinity is poor, it is possible that the antibody might break away when presented with more abundant antigen e.g: reticulin in the rat composite block. A possible remedy is to adsorb the antigen onto insoluble vehicles e.g: antigen coated Sephadex beads, which can be effectively removed following absorption.

The finding of a variety of immunofluorescent patterns with ARA may be a further explanation for the failure to absorb the antibody with gluten. This variety of patterns indicates that there is a spectrum of antigens involved. It may be that a moiety in gluten which cross-reacts with reticulin initiates damage with release of secondary antigens from the damaged reticulin fibres. These secondary antigens - which could previously be 'concealed' - may subsequently become immunogenic and induce production of antibodies which react with reticulin fibres, but not with the primary noxious moiety in gluten.

The specificity and validity of the positive absorptions also need to be critically assessed. It would appear that the absorption was not due to non-specific uptake by the gluten of antibodies in general, for in parallel studies, other autoantibodies like ANF were not affected. A further control experiment which perhaps should have been performed, was to absorb the ARA positive sera with a substance of similar physico-chemical and structural makeup to gluten.This experiment was not performed for the reason that such a substance could not be found.

The variation of ARA titres with dietary treatment is highly unusual for an autoantibody, and in discussing this, Brown et al. (1973) point out that gluten can act as a hapten in the production of ARA. Allison et al. (1971)have postulated that haptens may have a role in the production of autoantibodies. Thus, if in an animal sensitized againsst a hapten, the hapten coupled to host cell or protein is injected into the animal, antibodies against the host cell or protein may be produced. Weigle (1965)

produced autoantibodies to thyroglobulin in this way. In coeliac disease there is evidence of sensitization of the host to gluten as shown by in vitro lymphocyte transformation with gluten (Housley et al. 1969). In sensitized patients gluten may become coupled to a host component i.e: reticulin, and this would result in production of ARA. Thus, presuming that the presence of hapten is necessary for the continued production of antibody ARA will disappear with gluten withdrawal.

This arguement leads to a second possibility to be considered in the production of ARA - that they may result from damage to the small-intestinal mucosa. This could arise in two ways. As discussed already. an immunological reaction may be initiated, involving reticulin. Alternatively, following small bowel damage, the mucosal surface may allow absorption of antigens which would normally be excluded, such as reticulin in the diet. This probably occurs with other dietary antigens e.g: ovalbumin in coeliac and other gastrointestinal diseases (Taylor et al. 1961, Kendrick and Walker-Smith 1970). It is unlikely however that this mechanism is mainly responsible for the production of ARA in gluten-sensitive enteropathy, for ARA is not a prominent feature of other gastrointestinal diseases with mucosal changes very similar to or morphologically indistinguishable from the lesion found in gluten-sensitive enteropathy. Thus, ARA was not found, except in one patient, in a large series of tropical sprue in this study, nor in children with post-infective malsbsorption. Furthermore, von Essen et al (1972) in their study of 20 children with malabsorption due to cow's milk intolerance noted that ARA was conspiciously absent. They considered that ARA was not an immunological marker of destruction of the intestinal mucosa.

ARA however, was found in a significant incidence in patients with Crohn's disease where there is a mucosal abnormality thought not to be due to gluten-sensitivity. In the study reported here and in the studies of Alp and Wright (1971) and Magalhaes et al. (1974) the incidece of ARA in Crohn's disease was about 25% although the titres were low. Unlike coeliac disease, the incidence of dietary antibodies, including antibodies to gluten fraction-3 (Taylor et al. 1964) is conspiciously low

in Crohn's disease and this militatesagainst the ARA being the result of antigens absorbed through a damaged mucosa. Alp and Wright (1971) therefore suggest that ARA in this disease may be thae direct consequence of tissue damage in the intestine. Alternatively, the antibodies may be responsible for the perpetuation of the lesion which in Crohn's disease might be initiated in some other way, at present unknown.

ARA in Other Diseases

Ara was predominantly found in patients with D.H. and with coeliac disease. The only other condition with a significantly increased incidence was Crohn' disease in which it was found in 9 (24%) of the 38 patients investigated here. The titres however were low - 1:10 in 7 and 1:20 in 2. Alp and Wright (1971) found the antibody in 25% of 59 patients and a similar incidence was reported by Magalhaes et al. (1974)

In the other 534 sera investigated in this study - from patients with dermatological, gastrointestinal and immunological disorders, ARA was detected in only 8 patients, an incidence of about 2%. Alp and Wright (1971) however found a higher incidence in the 583 sera they tested - 13% of 33 patients with aphthous ulceration, 8% of 106 patients with ulcerative colitis, 8% of 72 patients with uveitis and 8% of patients with other miscellaneous disorders. They found an incidence of 4% in 68 normal adults but none in 43 normal children. These workers however, tested for ARA using a 1:5 dilution of the serum, whereas in this thesis the initial dilution used was 1:10. This difference and the interpretation of atypical patterns may have contributed to the discrepancy.

In the study of Brown et al. (1973) a much higher incidence (15%) of ARA was found in 48 control children. Rizzetto and Doniach (1973) in their study of 3542 sera from healthy subjects and from patients with various diseases found an incidence ranging from 4% in normal healthy adults to 20% in chronic heroin addicts. These workers however, included in their study immunofluorescent patterns given by various cellular components. In this thesis, such miscellaneous patterns were specifically excluded and it is likely that they account for the differences.

In the study of Magalhaes et al. (1974) ARA was noted in only one patient with chronic liver disease and in one patient with chronic lymphatic leukaemia. All 25 normal adults investigated were negative.

In one other group, an increased incidence of ARA has been noted. These are children with selective IgA defeciency. In the report of Alp and Wright (1971), 4 children with coeliac disease and selective IgA defeciency had ARA. Amman and Hong (1971) reported an antibody to basement membrane in 3 children with coeliac disease and selective IgA defeciency, in a study of 31 children with IgA defeciency. von Essen et al. (1972) found ARA in 6 of 26 patients with selective IgA defeciency. In two of these, small-intestinal showed total villous atrophy compatible with coeliac disease. The other 4 had normal biopsies. In these four, it is possible that the ARA was indicative of defective antigen handling by an IgA defecient small intestine.

Specificity of ARA as a Diagnostic Test

The foregoing discussion indicates that ARA, though prominent in patients with gluten-sensitive enteropathy, is not specific for this disease. It was found in about 25% of patients with Crohn's disease and in 8 of 534 non-D.H. or coeliac subjects investigated in this thesis. As a diagnostic test, it is most specific in identifying children with coeliac disease on a normal diet, whilst none of the childhood control sera were positive. The usefulness of this was borne out by the finding of ARA in a child investigated in a preliminary investigation in whom the diagnosis was not initially established, but who was thought not to have coeliac disease. Subsequent intestinal biopsy, performed as the result of finding ARA showed changes typical of coeliac disease. On treatment with a gluten-free diet, she made a good recovery. ARA is probably most useful as a diagnostic test in this age group - hence, the positivity is high in coeliac disease patients and low in patients without the disease.

von Essen et al. (1972) considered that as a discriminant, ARA was most useful in differentiating malabsorption due to cow's milk allergy

from coeliac disease. Both these conditions are assciated with abnormal jejunal biopsies indistinguishable by morphology. Thus, in 20 children with cow's milk allergy investigated by these workers, ARA was completely absent, wheareas in 40 children with coeliac disease 27 (68%) had the antibody. They considered ARA to be of practical value and suggest that if a patient had ARA he should be assumed to have coeliac disease and treated accordingly with a GFD.

A similar though less convincing picture is seen in adults with coeliac disease, where the incidence was about 60% in patients on a normal diet. Titres however, were generally lower and the positivity in non-coeliac adults higher. ARA is therefore a poorer discriminant in this group. With D.H. patients, the test appeared to have little value as a first line diagnostic aid since it was found only in 30% of patients on a normal diet.

The range of the normal incidence of ARA - from 0% to 15% - reported by various workers is remarkable and should be examined in some detail. It is likely that the main reason for the discrepancy is the different criteria used by the various workers in defining a serum as being positive for ARA. This is a problem obviously needing stricter definition and it is hoped that this will be resolved in the near future by cooperation between workers in the field. A second, though probably more minor contributory factor is the use of different groups of patients in each study. The situation will no doubt become clearer with the study of more sera from other disease groups.

AUTOANTIBODIES IN D.H. AND ADULT COELIAC DISEASE

The main finding was the increased incidence of antinuclear factor (ANF - anti-nuclear antibodies) in patients with D.H. In ACD however, the incidence was not raised. In addition, gastric parietal cell, thyroid microsomal and mitochondrial antibodies were found in a number of D.H. and ACD patients.

The implications of these findings however, is more elusive, as the full significance of autoabtibodies is notunderstood at the moment. It is probable that they arise as a result of immunological reaction to self antigens, though the stimulation for this self autosensitization

is not known. Autoantibodies are useful as diagnostic aids and their presence dilineate a definite group of diseases in which immunological phenomena are prominent.

Hence, in systemic lupus erythematosus, the incidence of ANF is almost 100%. In this disease, considered to be the prime example of autoimmunity and immune complex disease, definite immunological abnormalities are recognised. These include the high incidence of antibodies to native (double stranded) DNA (Arana and Seligmann, 1967) and low total serum complement and components of complement. In addition, immunoglobulin and complement deposits have been found in the kidneys - where they have been shown definitely to be DNA complexes (Koffler et al. 1967) - and in the skin (Burnham et al. 1963).

Increased incidences of ANF have also been found in patients with rheumatoid arthritis, scleroderma and other diseases associated with autoimmunity, including chronic active hepatitis, primary biliary cirrhosis and myasthenia gravis. The indications are that raised incidences of ANF reflect disturbances or impairment of immune function and the finding of a raised incidence in patients with D.H. in this study suggests that there may be a wider or more general immunological disturbance in D.H. than in coeliac disease. As to whether this discrepancy contributes to the differences in these two diseases remains to be seen.

The finding of an increase in the incidence of gastric parietal cell and thyroid microsomal antibodies in D.H. confirms the findings of Fraser (1970). An increase in the incidence of antibodies to thyroglobulin noted by Fraser (1970) however, was not seen in this thesis.

The association of D.H. with thyrotoxicosis has been

previously noted (Du Castel 1912; Curth 1955; Smith 1966; Kumar 1973). In addition, pernicious anaemia has been found in association with D.H. (Ilyas 1968; Cream and Scott 1970; Kumar 1973). In the series of 56 patients with D.H. reported in this thesis, 3 had a history of thyrotoxicosis and 2 of pernicious anaemia. All these five patients were in the original group of 29 D.H. patients investigated for autoantibodies.

Smooth muscle antibodies were found in equal proportions in both D.H. and adult coeliac disease patients. The incidence however, was not raised when compared with the normal healthy controls. A raised incidence was observed in the childhood coeliac disease group and in children with other miscellaneous gastrointestinal disorders, and in patients with vitiligo. Smooth muscle antibodies are now thought to be directed against antigens in cell membranes (Holborow 1972). As markers of autoimmunity they are found most frequently in patients with liver disease (Holborow 1972).

Increased incidences of smooth muscle antibody have also been reported in various viral illnesses such as viral hepatitis (Farrow et al. 1970) and in infectious mononucleosis (Holborow et al. 1973). It has been suggested that such antibodies might reflect viral damage to cell membranes and hence a viral pathogenesis (Holborow 1972). If this is the case, then the findings of normal incidences of smooth muscle antibody in D.H. and adult coeliac disease do not support a viral aetiology for these diseases.

In a study of autoantibodies to jejunal tissue, Dick et al. (1969) found antibodies of low titre reacting against the basement membrane zone of jejunal mucosa in 8 of 18 patients with D.H. Some of the sera reacted with normal jejunal mucosa and some with abnormal mucosa. In addition, they found fixed immunoglobulin deposits, detected by immunofluorescence

in the basement membrane zone of jejunal mucosa from 4 D.H. patients. These findings, however, were not confirmed in the study of Holubar et al. (1971). In coeliac disease, antibodies against the cytoplasm of jejunal epithelial cells were reported by Malik et al. (1964). Rubin et al. (1965) however, were unable to confirm this finding.

DIRECT IMMUNOFLUORESCENCE ON SKIN FROM D.H. PATIENTS

Immunoglobulin Deposits

The results reported in this thesis indicate that IgA is almost always present in D.H. skin. Of particular note was the finding of these deposits in the clinically uninvolved skin, even in those patients whose skins were completely controlled, either with dapsone or a gluten-free diet. In fact, these IgA deposits were generally absent if the biopsy was taken from a late blister. This is now a well established finding (van der Meer. 1972; Jablonska et al. 1973) and probably accounts for the initial negative reports of Jordon et al. (1967) and Chorzelski and Cormane (1968) who only studied involved skin. For this reason, the studies in this thesis have been largely confined to the investigation of clinically uninvolved skin, or where lesions are present, to early erythematous and peri-bullous skin. These latter biopsies generally gave similar patterns of immunofluorescence to those seen with clinically uninvolved skin.

Cormane (1967) was the first to describe the presence of immunoglobulins in the uninvolved skin in D.H. The class of immunoglobulin however, was not reported. Subsequently, van der Meer (1969) reported the presence of IgA in the uninvolved skin of 10 of 12 D.H. patients. This has since been confirmed by various other studies (Chorzelski et al 1971; Holubar et al. 1971; van der Meer 1972).

IgA was found in the clinically uninvolved skin in all 50 D.H. patients investigated in this thesis. Of the 80 miscellaneous skin biopsies from these patients, 78 were positive for IgA. The other 2 were completely negative for IgA, IgG and IgM.

Two distinct patterns of immunofluorescence of IgA deposition were seen in the uninvolved D.H. skin biopsies. In the 'papillary' pattern, the immunoglobulins were found only in the dermal papilla. This coincides with the granular, microgranular and fibrillar deposits described by Chorzelski et al. (1971) and van der Meer (1972). It is probable that 'papillary' is a better description as it localizes the site of IgA deposition, whereas the other terms only refer to the appearance of immunofluorescent staining seen. As will be discussed later, it is probable that these IgA deposits are located on the reticulin fibres of the dermal paillae and the appearance of either 'fibrillar' or 'microgranular' fluorescence probably depends on the plane of section of the fibres i.e: whether they are cut transversely or longitudinally.

The second, and less common pattern of immunoglobulin deposition seen in D.H. skin is the 'continuous'. This was previously described by Chorzelski et al. (1971) but was not found in the study of Holubar et al. (1971). Van der Meer (1972) too did not find the continuous pattern, but he stated that 'occasionally, the granular staining was found to occur continuously at the junctional zone of the skin sections'. The continuous pattern follows the line of thebasement membrane, but on further study using immunoperoxidase electron microscopy, the immunoglobulin deposits were shown to be beneath the intact basement membrane. (Seah et al 1972). This is distinct from pemphigoid where the immunoglobulin deposits are on

the basement membrane.

Using histochemical stains, the closest identification obtained with these IgA deposits was with reticulin detected by silver staining, and it is probable that the immunoglobulins detected by immunofluorescence are located in the reticulin fibres of the dermal papillae. No correlation was seen with stains for collagen, elastin or any other cell type.

The most common pattern of IgA deposition was the 'papillary' - seen in 68 (87%) of the 78 positive biopsies in this study. 'Continuous' IgA was seen in 10 (13%) of the 78 biopsies. These 10 biopsies came from 6 patients. In the study of Chorzelski et al. (1971) the incidence of the continuous pattern was much higher - in 5 of their 19 patients.

IgA was found in all the 78 positive biopsies. In addition to IgA, IgM was found in 7 biopsies and IgG in 2. In one biopsy, both IgG and IgM were present in addition to IgA. Chorzelski et al (1971) also found IgM and IgG in addition to IgA in about 20% of their 19 patients whilst van der Meer (1972) found IgM and IgG in about a quarter of his 22 patients. Holubar et al (1971) however, did not find IgM or IgG in any of their patients.

In this study, in the 9 biopsies which had IgG or IgM in addition to IgA, the pattern of deposition of the two classes of immunoglobulin was the same in 8. In the remaining biopsy in which there was a difference, IgA was present in a continuous pattern and IgM in a papillary pattern. In the single biopsy in which all three immunoglobulins were present, the IgA was papillary and the IgG and IgM both continuous. In the 15 patients who had two biopsies from the uninvolved skin at different times, 6 had had different results. In 2, the biopsy was repeated because no immunoglobulins were found in the first biopsy, but was on the second. In the remaining four, there was a difference in the class of immunoglobulins detected; in 3 there were also different patterns of immunoglobulin deposition in the first and second biopsies.

It would appear that IgA is a constant feature, but that there is otherwise no consistency in either the pattern of staining seen or the immunoglobulin class. It is not possible to explain these differences at the present time,

but they do not appear to be related to differences in the clinical features of the disease, age or sex of the patients, or in treatment at the time of biopsy. In addition there was no difference in class or pattern of immunoglobulin deposition between those patients whose rash was being controlled by a GFD, those being controlled with dapsone and in those not receiving any treatment.

Value of IgA as a Diagnostic Criterion

The incidence of IgA in the clinically uninvolved skin was 100% in the series of 50 D.H. patients studied here. A similar incidence was also found by van der Meer (1972) in his study of 22 patients. Chorzelski et al (1971) found IgA in all their 19 patients with 'typical' D.H. but in none of their 8 patients with 'atypical' D.H. These high incidences indicate that IgA in the clinically uninvolved skin should now be seriously considered as a major diagnostic criterion in the diagnosis of D.H. As has been elaborated in the introduction to this thesis and elsewhere (van der Meer, 1973) the definition of D.H. has yet to be resolved and disagreement in terminology, especially in relationship to pemphigoid, has plagued study in the field for many years. Smith (1966) stated that there is no 'universal agreement as to the criteria for making the diagnosis of D.H.'

With this in mind, in a recent study, Fry and Seah (1974) examined the diagnostic criteria for D.H. and attempted to evaluate the relevance of more recent findings, such as IgA in the skin, in this context. They examined 42 patients in whom a diagnosis of D.H. was made by nine consultant dermatologists over a period of six years. The diagnostic criteria they examined were :

1 - Clinical features
2 - Response of rash to dapsone or sulphapyridine and clinical relapse on withdrawal of therapy
3 - Histology of skin lesions
4 - Anti-reticulin antibody
5 - IgA in the uninvolved skin
6 - Small intestinal biopsy
7 - [illegible]

Of these 42 patients, the diagnosis of D.H. was substantiated in 35. All these 35 patients had IgA in the clinically uninvolved skin and the authors came to the conclusion that the presence of these IgA deposits was the best guide to diagnosis and that in their absence, the diagnosis should not be made.

Abnormality of the small intestine was also a good diagnostic criterion, being positive in 34 of the 35 D.H. patients but only in 1 of the 7 non-D.H. patients. Clinical features, histology, ARA and folate status were found to be poor diagnostic criteria. The response to dapsone or sulphapyridine was present in all 35 D.H. patients, but as it was also positive in 6 of the 7 non-D.H. patients, it was a poor discriminant. This test however, is otherwise useful in that if there is no response, it almost certainly excludes the diagnosis of D.H.

The frequency, site and pattern of immunoglobulin deposition and the class of immunoglobulin involved are now being used increasingly for diagnostic purposes and in helping to understand the pathogenesis of various skin diseases. IgA in the clinically uninvolved skin, other than in D.H. is a rare phenomenon. Baart de la Faille-Kuyper et al. (1973) described IgA deposits in the uninvolved skin of patients with Henoch-Schonlein purpura but these were in the blood vessels in the skin and quite different morphologically from the deposits seen in the D.H. skin. IgA in the skin has been inconstantly found in patients with pemphigoid, LE (Cormane et al.1966) and more recently in lichen planus (Michel and Sy, 1973). However, as in Henoch-Schonlein purpura, the patterns of immunofluorescent staining were quite different from those seen in D.H. and the IgA was usually found in addition to IgG or IgM (which were more constant features). Moreover, these deposits were found more frequently in involved skin. The papillary pattern of IgA in the skin in D.H. appears to be distinctive for the disease and has not been reported in other skin diseases. The continuous pattern however, has given rise to confusion and to reports that it is difficult to distinguish pemphigoid from D.H. (Honeyman et al, 1972). This confusion is understandable if only the pattern of immunoglobulin deposition is considered. However, if the

immunoglobulin class is taken into account, the picture becomes clearer. In D.H. the continuous band in invariably IgA. Only one of the six patients with continuous IgA in this study had IgG in the same position. In pemphigoid on the other hand, the continuous (basement membrane) band is invariably IgG and occasionally IgM or IgA. Hence Beutner and Chorzelski (1973) did not find any IgA in the bullae of patients with pemphigoid and in the 7 patients of Dr. R. Jordon cited by the same authors, only one had IgA deposits. All 7 however, had IgG deposits and one IgM as well. However, it was not stated as to whether involved or uninvolved skin was examined.

Further differentiation between D.H. and pemphigoid can be made by serological studies which show that about 85% of patients with pemphigoid have circulating basement membrane antibodies. None of the 7 patients in this study with continuous deposits of IgA had basement membrane antibodies.

A recent report by Holubar et al (1973) of Ig A in addition to IgG in a continuous pattern in the skin of 2 of 5 patients with cicatricial pemphigoid (CP) is of interest. Bean and Michel (1973) however, could only find IgG in 5 of their 6 patients in the involved skin or mucous membrane. In the uninvolved skin, only 1 of the 6 was positive. In the study of Holubar et al (1973) the biopsies were from involved or previously involved skin. It is reasonable that in diseases affecting the mucous membrane, as in CP, IgA should be present as part of the pathology, for IgA is the main immunoglobulin involved in the immune responses of mucosal surfaces, including the buccal cavity and gastrointestinal tract. A corollary exists in the finding of IgA in the skin in D.H. - a disease known to be associated with an abnormality of the small intestine. The situation in CP needs to be clarified with further study. It would appear that, as with pemphigoid, continuous IgG deposits are usually present and IgA only occasionally. It is also conceivable that D.H. and CP can exist together. In the series of D.H. patients studied in this thesis one patient had unequivocal evidence of both diseases.

The low incidence of IgA in skin diseases in general is substantiated in this study by the complete absence of IgA in all the 54 skin biopsies from 48 non-D.H. patients, investigated by immunofluorescence. A similar situation was found in the skin biopsies from 21 patients with adult coeliac disease and the 9 volunteers.

In summary, it would appear that IgA in the clinically uninvolved skin is a good and specific test for diagnosing D.H. and it is suggested that this should be used as the main diagnostic criterion. The main advantage that this test offers is specificity. In addition clinically uninvolved skin is all that is required. Hence, the patient can be completely controlled with dapsone, removing unnecessary discomfort to the patient by stopping therapy and waiting for relapse of the skin rash. There is also no need to look for suitable lesions which are usually difficult to find, especially in chronic and longstanding cases.

One point arising from this study is that, very occasionally, IgA can be absent in the uninvolved D.H. skin. Thus in 2 of the 50 D.H. patients studied here, IgA was not found in the initial skin biopsy. Further biopsies were performed on both patients as the index of clinical suspicion was high - and in both, they were subsequently positive. This point has also been made by Jablonska et al. (1973) who found that IgA can be missed on a single section or biopsy. Hence, serial sections were required in 3 of their patients to detect IgA and in a further patient, a second biopsy was necessary after 30 consecutive sections of the first biopsy had failed to reveal IgA.

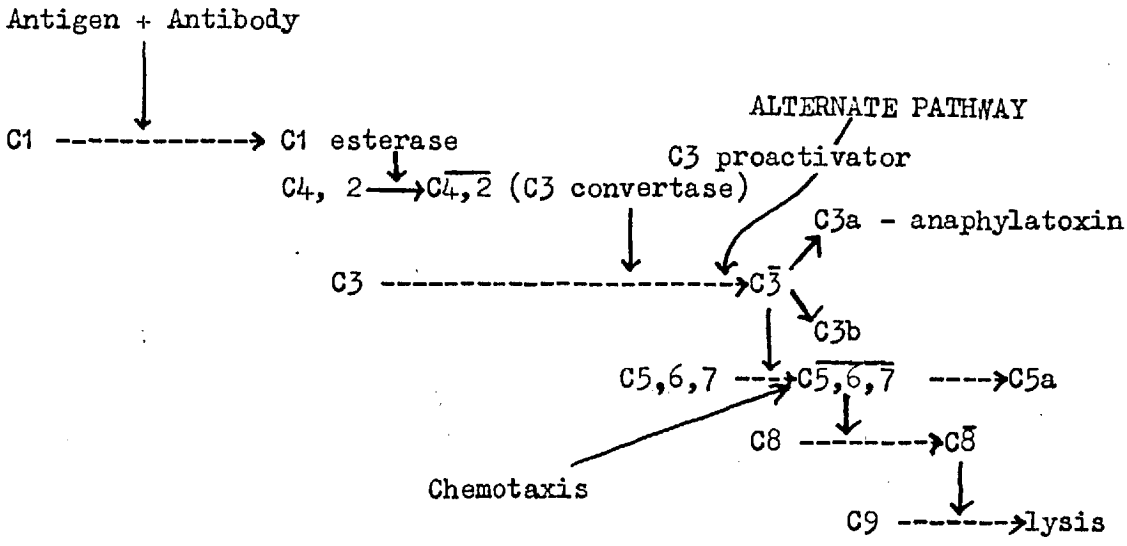
COMPLEMENT STUDIES

The studies reported here indicated that complement, detected as C3 deposits, was present in the DH. skin. 50% of 52 biopsies examined had C3 deposits. When these were related to the sub-groups of skin biopsies, the highest incidence was found in the involved skin of D.H. patients on no treatment (12 of 13 biopsies). In the uninvolved skin of patients on no treatment, only 3 of 12 biopsies were positive. In patients controlled with dapsone, 8 of 19 biopsies were positive and in the patients controlled on a GFD alone, 3 of 8 biopsies were positive. In all these 52 biopsies, IgA

was present and the pattern of C3 deposition corresponded in all cases to those given by the IgA deposits.

The association of C3 with the active lesions strongly indicate a close relationship between disease activity and complement and suggests that complement is activated in the production of the D.H. skin lesion.

Complement is usually activated following reaction between antigen and the appropriate complement-fixing antibody. This triggers off the complement cascade and the subsequent events are briefly illustrated below:-



The events occuring after complement fixation are well recognised and are classically seen in the Arthus-type reaction (Type III reaction, Coombs and Gell, 1969). These events essentially mediate a variety of effector mechanisms that follow antigen-antibody reaction. Inflammatory changes are initiated and it would be relevant to discuss here the changes seen in the early D.H. lesion in this context. Histologically, the main finding in the D.H. lesion are (a) microabscesses which subsequently coalesce to form a large subepidermal blister (b) infiltration with polymorphs and eosinophils and (c) fibrin deposition (Mustakallio et al, 1970; Connor et al, 1972). When these features are related to the inflammatory changes occuring in tissues after complement activation, a good correlation is obtained. Infiltration with polymorphs and eosinophils is a well recognised feature of the D.H. lesion (Pierard and Whimster, 1961). In the complement activation sequence, such chemotaxis is seen following the activation of

C5, 6 and 7. In addition, the C3a and C5a anaphylatoxins released contribute to the chemotaxis and histamine release. The total effects of these factors result in increased capillary permeability and the outpouring of fluid into the surrounding tissue. In D.H. such an event could lead to the formation of microabscesses in the dermal papilla and suggests that this is the primary site of involvement in the formation of blisters. The microabscesses subsequently coalesce, the result of stretching and necrosis of the inter-papillary ridges which finally dissolve or rupture to give rise to a large sub-epidermal blister. The deposition of fibrin is a well recognised accompaniment of complement activation and is seen abundantly in the D.H. lesion (Mustakallio et al, 1970). Kaplan et al (1971) have reported evidence to suggest that antigen-antibody complexes are capable of activating the clotting system via activation of Hageman factor (Factor XII). Another mechanism for fibrin deposition has been reported in rabbits by Zimmerman and Müller-Eberhard (1971) who demonstrated a Hageman factor dependent pathway of coagulation dependent upon activation of the complement system.

The localization of these inflammatory reactions to the dermal papilla, as has already been mentioned, is of great interest, for it is in this situation that the IgA and C3 deposits are found. These findings suggest that IgA and C3 are very closely involved in the pathogenesis of the DH lesion and the histological studies supports the concept that the damage is complement mediated.

This concept however, is a little surprising when one considers that the main, and frequently only immunoglobulin class involved is IgA. IgG and IgM antibodies are both well-recognised complement fixing antibodies, but not IgA. However, this is only when one considers the "classical pathway" of complement activation where all the complement components (C1 to C9) are involved. It is now recognised that a second pathway of complement activation - the "alternate pathway" - exists, where the first three components of the classical pathway, C1,2, and 4 are bypassed, and activation of C3 is the initial event in the triggering off of the complement sequence (Sandberg

et al, 1970; Gotze and Müller-Eberhard, 1971). This pathway may be initiated by various non-immunological substances like endotoxin, lipopolyssacharides, zymosan and cobra venom. In addition, aggregated immunoglobulins, including IgA can act in the same way (Spiegelberg and Gotze, 1972).

With this in mind, the experiments using anti-C3 and anti-C1q were performed on the D.H. skin biopsies to elucidate if such a mechanism could be involved in the pathogenesis of the D.H. lesion. C1q is a subunit of C1 and its presence with C3 in a lesion suggests complement activation by the classical pathway. In the 34 biopsies studied, IgA was present in all, IgM in 9 and IgG in 3. C3 deposits were found in 16 (47%) of these 34. C1q deposits however, were found in only 3 (9%) and in all these 3, IgG or IgM deposits were also present in addition to the IgA. This conspicious absence of C1q suggests that in the skin in D.H., C3 is activated by the alternate pathway. In those biopsies where C1q was present, classical pathway activation was probably initiated by the IgG or IgM also present. Absence of C1q however, does not necessarily exclude classical pathway activation, for after activation of C4 and C2 to form C3 convertase, C1 may dissociate from the complex (Rapp and Borsos, 1970).

From these observations the question of the role of IgA arises. The close association of IgA with the C3 deposits suggests that IgA is the agent responsible for activating complement in the skin in D.H. If this is the case, the IgA is presumably in a modified form - either aggregated, or possibly complexed in some way which makes it activate complement. The studies using anti-IgA secretory piece performed in this thesis indicate that secretory IgA is not involved.

The finding of C3 in the uninvolved skin in the other D.H. patient-groups requires explanation. In the uninvolved skin of patients not on therapy, it is highly probable that the C3 deposits are located in very early lesions not macroscopically visible. In the patients controlled on dapsone only, it is possible that the dapsone might not block complement activation, but acts by affecting a subsequent process in the formation of the D.H. lesion.

Hence, it may block the inflammatory and chemotactic factors released following complement activation. This is consistent with the observation of Fry et al.(1973) that, at electron microscopic level, membrane-bound vacuoles are present in the uninvolved skin of patients controlled on dapsone. These vacuoles were considered to be an early stage in blister formation (Riches et al, 1973) and presumably occur after complement activation if such a mechanism was operative. The finding of C3 in the skin of patients whose rashes were controlled by a GFD alone is perhaps the most difficult to explain. It is possible that in this group, C3 activation is still taking place, but not at a level sufficient to produce lesions clinically; alternatively, the C3 could be in an inactive form and that activation is dose-related to the quantity or nature of IgA present. In this context, the removal of gluten from the diet may be the factor responsible.

Studies by other workers also indicate that in DH alternate pathway complement activation takes place in the skin (Provost and Tomasi, 1973, 1975; Rogers and Jordon, 1973). Provost and Tomasi (1973) showed more convincing evidence than the mere absence of C1q by demonstrating the presence of C3 proactivator and properdin in the skin lesions. These two factors are believed to be specific for alternate pathway complement activation. In addition they demonstrated the absence of C4. Evidence for alternate pathway complement activation has also been demonstrated in several other human skin conditions including bullous pemphigoid, LE and herpes gestationis (Provost and Tomasi, 1973) whilst IgA in the dermal capillaries with C3 have been found in the involved and uninvolved skin of patients with Henoch-Schonlein purpura and Berger's nephritis (Tsai et al.1975). Other reports of C3 in the skin in DH. have been reported by Cormane (1967), Cormane et al.(1970), van der Meer (1969, 1972) and Holubar et al.(1971). In the report of van der Meer, (1972), complement deposits were found in all 22 D.H. patients studied.

In a study of serum complement (as C3) in 10 patients with D.H., Fraser et al. (1971) could not detect any significant abnormality and

concluded from this that there was no further evidence for an immunological mechanism in D.H. This is not necessarily a correct conclusion for the serum complement level reflects a balance between complement synthesis and degradation and it is possible that synthesis of complement was sufficient to replenish that lost by consumption during active disease. A normal level of complement therefore does not militate against an immunological pathogenesis.

Histocompatibility Studies

The results of this study show that in both D.H. and A.C.D., the incidences of HL-A 1 and HL-A 8 separately, and of HL-A 1 and 8 together are raised. However, as already discussed (page 138), the significant abnormality was a rise in the incidences of HL-A 8 and it would appear that this raised incidence is the abnormality most likely to be associated with gluten sensitivity.

The finding of an incidence of 77% for HL-A 8 in patients with D.H. is higher than in any previous study. Katz et al. (1972) reported an incidence of 58%, White et al. (1973) 60% and Gebhard et al. (1973) 68%. The incidence of HL-A 8 in the control group was similar to that found in the previous studies, and thus the greater incidence of this antigen in the D.H. patients cannot be explained by a difference in the populations. It is more likely that the criteria adopted for diagnosing D.H. in the present study were more restricted as they included the presence of IgA in the uninvolved skin. A small number of patients reported in the previous studies may not, in fact, have suffered from D.H., within this definition and hence not shown the genetic association. Indeed, Fry & Seah (1974) have shown that if only the criteria of clinical and histological features, and response of the rash to dapsone are used for diagnosis as in the studies of Katz et al. (1972), White et al. (1973) and Gebhard et al. (1973) it is possible that up to 15% of patients may not have the disease. If the assumption is made that 15% of patients in these other studies did not have D.H., the incidence of HL-A 8 would be increased to about the incidence reported here.

The incidence of 77% of HL-A 8 in patients with D.H. is not significantly different from 88%, the incidence of this antigen found here in A.C.D., and is similar to that reported by other workers in A.C.D. - 88% by Stokes et al. (1972) and 87% by Falchuk et al. (1972).

Recently, Gebhard et al. (1973) have suggested that there are two groups of patients with D.H., one having gluten sensitive sateropathy (GSE) and showing a similar incidence of HL-A 8 (84%) to patients with A.C.D., and a second group who do not have G.S.E. and who do not show an incidence of HL-A 8 greater than controls. Gebhard et al. divided their group of 28 D.H. patients into 19 who showed macroscopic and histological evidence of an enteropathy, and 9 patients who did not show evidence of an enteropathy. The criteria they used to define an abnormal macroscopic or histological appearance were not given. In this present study, this division was not bourne out for there was no significant difference in the incidence of HL-A 8 between the D.H. patients whose biopsies had an abnormal macroscopic appearance and those with a normal appearance. (Table IV, 19). This was also found to be so in the study of White et al. (1973). It may be possible to reconcile the findings in the two studies if a small number of patients in the series by Gebhard et al. (1973) did not have D.H. If one applies the figure of 15% for misdiagnosis mentioned above, it is possible that 4 of the 28 patients in their study did not have the disorder. If this is so, these 4 patients would almost certainly belong to the group of 9 patients in whom they found no enteropathy. It is then possible that the incidence of HL-A 8 in the remaining 5 patients in this group would be similar to that in their group of 19 patients with enteropathy.

A feature of the tissue typing in A.C.D. and D.H. was the high incidence of the 4b antigen which occurred in all 36 A.C.D. and 38 of 39 D.H. patients studied. It was decided to test for the 4a and 4b antigens in this study in view of the known frequent association with the HL-A 1 and 8 chromosome. Despite the high frequency, analysis of the results here indicate that it was due to this association and was not a significant abnormality. The

failure to find HL-A 8 or 4b in the Egyptian patient may be explained by a different racial frequency of antigens as the incidence of HL-A 8 in the South-east Mediterranean basin and Ethiopia is low.

Increased frequencies of certain HL-A antigens have now been described in a large number of diseases. The full significance of these increased incidences is at the present time not fully apparent. It is possible that they might be associated with 'immune response' (ir) genes, which control the ability of an individual to respond to antigenic stimulation (McDevitt & Bodmer 1972). Thus, in animal studies, an association between the major histocompatibility system in the mouse (H2) and increased susceptibility to viral leukaemia has been demonstrated (Lilly et al. 1964, Tennant & Snell (1968). It is possible that in D.H. and A.C.D., the presence of HL-A 8 influences the 'ir' genes. However, this cannot be the whole explanation as not all patients with D.H. and A.C.D. possess the antigen.

A further suggestion is that HL-A antigens might cause disease because they share common antigenic determinants with certain infective agents, for example viruses (Snell 1968). The individual's immune system therefore recognises these agents as 'self-antigens' and does not mount an effective immune response, allowing the invading agent to proliferate and become pathogenic. There is as yet no direct or circumstantial evidence at the moment to suggest that viruses or other infective agents are implicated in the pathogenesis of D.H. or A.C.D. It has been shown that both the skin and intestinal lesions in D.H. are due to gluten (Fry et al. 1968, 1969, 1973, Barnetson et al. 1973) and the finding of raised incidences of HL-A 8 in D.H. and A.C.D. reflect further evidence of immunological abnormality in these disorders and suggests a common aetiology in their pathogenesis.

SMALL-INTESTINAL FINDINGS IN DERMATITIS HERPETIFORMIS

Macroscopic Appearances

In the series of 43 patients with D.H. studied here, the incidence of abnormality of the small intestinal mucosa judged by macroscopic appearance, according to the criteria of Booth et al (1962), on a single mucosal

biopsy was only 54%. Marks et al.(1966) who first reported an abnormal small intestinal mucosa in D.H., found that 9 (75%) of their 12 patients had abnormal mucosa macroscopically while Fraser et al.(1967) reported this abnormality in 7 (58%) of their 12 patients. Fry et al.(1967) found abnormalities macroscopically in only 4 of their 12 patients (33%). Brow et al (1971), using a multiple biopsy technique - where they took 6 - 8 biopsies from each patient - found macroscopic abnormalities in each of the biopsies in 15 of the 22 D.H. patients they studied, and an abnormality in some of the multiple biopsies in a further 6 patients. Taken together they were therefore able to demonstrate macroscopical mucosal abnormalities in 21 (95%) of these 22 patients. They infered from this finding that the mucosal lesion in D.H. waspatchy and mild compared with that found in patients with coeliac disease and that this may account for the conspicious absence of gastrointestinal symptoms in these patients (Fry et al. 1967; Marks et al 1968; Shuster et al, 1968). It is now well established however, that the enteropathy in D.H., although mild, is due to gluten sensitivity (Fry et al, 1967, Shuster et al, 1968; Weinstein et al, 1971).

Microscopic Appearances

At a microscopic level, the majority of D.H. small-intestinal biopsies showed considerable lymphocytic infiltration. This was most conspicious in the lamina propria, where in addition, increased numbers of plasma cells were found. Such increases in chronic inflammatory cells have been previously noted (Paulley, 1954; Rubin et al,1960; Anderson, 1960). When examined more closely, the infiltration with lymphocytes was seen most conspiciously in the mucosal epithelial cells. In that situation, it was easy to quantify the number of lymphocytes present by relating them to the number of epithelial cells present. In the lamina propria, the irregular morphology made such quantification technically difficult and was not persued in this study.

Increased lymphocytic infiltration of the small intestinal mucosa was previously noted by Ferguson and Murray, (1971) in patients with coeliac disease. The range of the counts in their controls was 6-40 per 100 epithelial cells which is higher than the 69-285 per 1000 epithelial cells reported

here. This discrepancy may be accounted for by variation in the method of quantification. When performing lymphocyte counts, it should be stressed, as was observed in the study of Ferguson and Murray (1971) that there are fewer lymphocytes towards the base of the villus compared to the top. Thus, counts performed mainly at the tip of a villus will tend to be higher. In an attempt to overcome this problem of variation, 1000 epithelial cells were counted in this study. This covered a total of cells approximately equal to the number of one side of six or more villi and therefore gave a more accurate estimate of the lymphocytic infiltration present.

Taking this lymphocytic infiltration as an index of morphological abnormality, it appears that the majority of the D.H. biopsies examined here are abnormal. The range of the lymphocytic counts in the control series was 69-285., mean 159 ± SE 13. In all the 4 D.H. biopsy groups the counts were considerably, and significantly, higher - irrespective of the macroscopic mucosal appearance. Hence, all but 3 of the 43 biopsies had counts well below the borderline level of 200 per 1000 epithelial cells. The most marked infiltration was found, surprisingly in the patients with macroscopically 'leaves only' mucosae. Such macroscopic appearances are considered by many to be normal and not indicative of coeliac disease.

The studies on the distribution of the intraepithelial lymphocytes in relation to the epithelial cell nucleus indicate that they be of use diagnostically. The number of cells in the basal (B) position was remarkably constant in both control and D.H. patients. The main increase appeared to be around thenucleus - perinuclear (PN) - and there were significant differences between the controls and all 4 D.H. groups. Supranuclear (SN) counts in the DH patients were also significantly increased but not to a degree seen with the PN counts. In the controls, the lymphocytes were distributed equally between the B and PN positions and there were virtually none in the SN position. The PN to B ratio was therefore approximately one in the controls, whereas in the D.H. biopsies, it was generally above 2, and in many cases higher (Figure IV. 43). This

PN:B ratio may be of use in distinguishing normal and D.H. biopsies with borderline total counts, as illustrated in Figure IV.44.

The degree of lymphocytic infiltration of the small bowel in D.H. appeared to be remarkably constant in those patients taking a normal diet. Hence, in the 5 patients who had repeat biopsies performed at intervals from 6 months to a year, there was little fluctuation in their total counts (Figure IV.45). In addition, the compartmental lymphocyte counts and PN:B ratios also remained constant. This, however, was not the case with the macroscopic appearances which showed fluctuations; (Figure IV.45).

The total lymphocyte counts however, fell with gluten withdrawal, indicating that the presence of gluten was responsible, at least in part, for the elevated counts. Hence, in the 13 patients who were treated with a GFD for periods varying from 1 to 4 years, all but one showed drops in the total count (Figure IV.46). The one remaining patient had counts within the normal range before and after treatment with a GFD. When related to the cell nucleus, the most pronounced fall was seen in the PN lymphocytes and to a certain extent in the SN lymphocytes (Table IV. 21). The total counts following treatment however, seldom fell to a range which could be considered to be normal. Hence, of the 12 biopsies showing improvement, only 4 returned to a figure below 200.

The fall in the intraepithelial lymphocyte count on gluten withdrawal was also noted in the studies of Ferguson and Murray (1971) and Holmes et al (1973). Both these groups also noted that despite the fall, the counts in the majority of patients after taking a GFD did not fall back into the normal range, despite treatment for many years. The failure of the counts to return to the normal range suggests that, despite gluten withdrawal, there is persisting (?antigenic) stimulation. Alternatively, it is possible that gluten initiates a sequence of events which perpetuates the stimulus despite the eventual removal of gluten. In this context, it is of interest to speculate as to whether the possible cross-reaction between gluten and reticulin, and the auto-antigenicity of reticulin, might contribute to this perpetuation.

In the 4 patients where studies involving re-introduction of gluten into the diet following treatment with a GFD, were done (Figure IV.47) - one patient showed a marked rise in the total count following gluten challenge. In a further patient there was a minimal rise whilst in the remaining 2 there was a fall. These results are not entirely convincing in suggesting that gluten is the stimulus for the increased lymphocytic infiltration. However, the number of patients examined was small and no definite conclusions can be drawn. Increase in the number of chronic inflammatory cells in the small-intestinal mucosa however, was noted in patients with coeliac disease following gluten challenge (Rubin et al. 1962; Shmerling and Shiner, 1970; Bayless et al. 1970).

The presence of these lymphocytes in the small bowel of patients with D.H. (and with coeliac disease) raises the question as to their significance. The results reported in this study and by others indicate that they are stimulated by the presence of gluten, and it is highly probably that this stimulus is immunological. There is evidence to show that patients with coeliac disease are sensitized to gluten - as demonstrated by lymphocyte transformation to gluten (Housley et al, 1969). In addition, Fakhri and Hobbs (1972) suggest that in coeliac disease, gluten antibodies 'co-opt' non-sensitized lymphocytes which then become pathogenic. Otto (1973) in a study of the lymphocytes in the small intestine found that in patients with coeliac disease, but not in normals, the ultrastructural appearances were those of activated lymphocytes ie: immunoblasts. Ferguson and Parrot (1972) have shown that a cell-mediated reaction can result in a lesion resembling coeliac disease in an experimental animal model. In addition, Ferguson (1974) has presented strong evidence to suggest that cell-mediated immunity, mediated by T-lymphocytes, is heavily implicated in the production of experimental villous atrophy in rats. Hence, in rats infected with Nippostrongylus brasiliensis the subsequent villous atrophy known to be produced by this infestation was abolised by previous neonatal thymectomy - a procedure known to deplete T-lymphocytes. (Ferguson and Jarrett 1975). Preliminary studies by Ferguson (1974) also suggest that the increased

intraepithelial lymphocytes in gluten-sensitive enteropathy are due to an increased flow of lymphoid cells into the coeliac jejunum rather than an intense local proliferation of lymphoid cells within the villi.

The increase of the lymphocytes in the perinuclear position was a prominent feature of the lymphocytic infiltration seen in this study. As to why this is so, however, is uncertain. It is well established from electron microscope studies (Meader and Landers, 1967; Toner and Ferguson 1971) that the lymphocytes in both the normal gut and in gluten-sensitive enteropathy are within the epithelial layer i.e: intraepithelial - but extracellular in relation to the epithelial cell. They are therefore properly called intercellular but not intracellular. In explaining the increased perinuclear counts in the D.H. small - intestinal biopsies, there are a number of possibilities. Firstly in the diseased mucosa, lymphocytes would theoretically be pushed away from the basement membrane by a purely mechanical effect - the lymphocytes being forced into a higher position simply because the basal region cannot physically accomodate more than a certain number. This, however, does not explain why more lymphocytes than usual are entering the intercellular spaces from the underlying connective tissue in the first place. No information is available on the relationship of the epithelial lymphocyte population to that of the lamina propria, but it is unlikely that the latter becomes so full of lymphocytes that they 'spill over' into the epithelium.

A second possibility is that the lymphocyte may rise to a higher position as a result of antigenic stimulation from gluten, either in the intestinal lumen or following absorption into the epithelial cell. Even when there is heavy infiltration and the epithelium remains columnar, there are relatively few lymphocytes in the SN position. This may be a purely mechanical effect, since although a considerable increase in the volume in the intercellular spaces is possible between the lower three-quarters or so of epithelial cells (as occurs for example during fluid absorption), the apices remain closely adjacent because of the concentration of intercellular attachments (as tight and intermediate junctions) in this area. In biopsies with cuboidal epithelium, hardly any lymphocytes fall

within the definition of supranuclear, since there is just not enough space between the top of the nucleus and the apex.

A third possibility is that in gluten-sensitive enteropathy, the epithelial cells, themselves may exert a chemotactic influence on lymphocytes, attracting them to a higher than normal position, perhaps as a result of substances produced by the epithelial cells under the influence of its own nucleus. The cell turnover in gluten-sensitive enteropathy is increased (Croft et al 1968) and presumably involves frequent and rapid nuclear replication. It is possible also that the lymphocytes are engaged in exchange of materials within the epithelium and vice-versa, and that the nearer the respective nuclei are to one another, the more quickly can the nucleocytoplasmic interchange and passage of information within each cell be effected. It is not known for how long the lymphocyte remains among the epithelial cells, but it must not be assumed that they all move up with the epithelial sheet to be shed into the lumen.

Three of the 43 patients with D.H. in this study had total lymphocyte counts below 200 per 1000 epithelial cells and could be taken to be definitely within the normal range. In the study of Ferguson and Murray (1971) one patient with coeliac disease had a normal count. One of the 3 D.H. patients with normal counts had a reticulum cell sarcoma of the small intestine removed (Goodwin and Fry 1973) and another has now developed an unexplained and progressive peripheral neuropathy, and the possibility of malignancy has to be considered. It is known that there is an increased incidence of small-intestinal lymphoma in patients with gluten-sensitive enteropathy (Gough et al, 1962; Harris et al, 1967) and it is possible that the low lymphocyte counts in the intestinal wall in some way reflects impaired immunity or immunological surveillancepredisposing to malignancy. However, it has also been suggested that the presence of excess chronic inflammatory cells in the mucosa in coeliac disease may predispose to the increased incidence of lymphoma. In the context of reduced immunological surveillance, the work of MacLaurin et al (1971) is of interest. These workers showed that lymphocytes from patients with coeliac disease had significantly reduced cytotoxic responses compared with lymphocytes from normal people, when

cultured with EB lymphoma cells. They infered from this that such impairment may increase the susceptibility of patients with coeliac disease to develop lymphoma.

The findings of increased lymphocytic infiltration in the small bowel in most of the D.H. patients studied here, taken together with those of Brow et al (1971) and Weinstein (1973, 1974) suggest that in D.H. virtually all patients have a gluten-sensitive enteropathy and that in approximately half of them the disease is so mild that there is no macroscopic abnormality in the small intestine, as judged on a single biopsy.

CONCLUSIONS

The small-intestinal findings in this thesis suggests that virtually all patients with D.H. have an enteropathy and do not support the concept put forward by Shuster et al (1968, 1970) and Gebhard et al (1973) that D.H. and coeliac disease are two distinct but geneticallylinked disorders. Instead, the results here suggest that D.H. should be considered as a single entity - a "unitarian concept" - in which the skin and gastrointestinal disorders are both part of the syndrome of "dermatitis herpetiformis". In addition to the small-intestinal findings, further support for this concept comes from the HL-A studies which show that patients with D.H. have a high incidence of HL-A 8 as found in patients with coeliac disease. There was in addition no difference in the incidence of HL-A 8 between the group with evidence of enteropathy macroscopically and those with no macroscopic abnormality. However, this latter arguement is, it is suggested, not tenable as the intraepithelial lymphocyte counts indicate that the macroscopic appearance alone may not be a sufficiently sensitive criterion in the diagnosis of gluten-sensitive enteropathy.

Quantitation of intraepithelial lymphocytes was in addition found to be a useful diagnostic aid as was comparing the position of these lymphocytes to the epithelial cell nucleus. The significance of these lymphocytes is uncertain, at the present time, but it is probable that they represent an immunological response to gluten.

IgA in the clinically uninvolved skin was found to be a very useful

diagnostic criterion and it is suggested that this should be adopted as the main criterion in making the diagnosis of D.H., and that in the absence of these deposits, the diagnosis should not be considered. Hence, IgA was found in all the 50 patients investigated and in none of the controls. It proved to be specific, especially when found in a 'papillary' pattern and was a good discriminant. It is stressed however, that IgA can be missed on the first biopsy, and that if the index of clinical suspicion is high, a further biopsy should be performed. The use of IgA deposits in the skin as a diagnostic criterion also has the advantage that completely uninvolved skin can be used and is of minimal inconvenience to the patient.

From an aetiological point of view, however, the significance of these IgA deposits is more elusive. They are present in the clinically uninvolved skin as well as in D.H. lesions. In addition, they remain in patients despite prolonged exclusion of gluten from the diet (up to 5 years). In this context, it is possible that the cross-reactivity between gluten and reticulin may be of relevance. Gluten could initiate the damage in the first place, and the cross-reactive reticulin may perpetuate the disease activity which persists (? by production of anti-reticulin antibodies) despite strict gluten withdrawal.

The IgA nature of the deposits suggest a direct link between the gut and the skin in D.H., for IgA is the main immunoglobulin involved in the gastrointestinal tract and is an unusual immunoglobulin to find in the skin. Taken together, the presence of these IgA deposits is further support for considering D.H. to be a single disease entity with dermatological and gastrointestinal manifestations.

It would appear that the IgA deposits are involved with the production of the skin lesions in D.H. for they were found in the very sites - the dermal papillae - where, based on histological evidence, it is thought that the initial events in the formation of the DH blister begins. Further support for this comes from the finding of C3 deposits, together with IgA in the dermal papillae. Complement activation appears to be involved, for

the C3 deposits were found in highest incidence in the early lesions of patients not taking dapsone. The histological features of the D.H. lesion also correlate well with themorphological events known to take place after complement activation.

The trigger to this complement activation however, is uncertain, but a number of possibilities can be considered. IgA is the main, and usually the only immunoglobulin involved in the D.H. lesion. IgA in an aggregated form is known to activate the alternate pathway of complement activation. The nature of the IgA deposits in the skin in D.H. is unknown, but it is unlikely that these deposits on their own are responsible for complement activation, for IgA deposits are present in normal skin and in patients who have no rash. An additional factor or stimulus, it would appear, is necessary, and a clue to this comes from the observation that a gluten-free diet can improve the rash. The presence of gluten in the diet is therefore the possible triggering factor. Following gluten ingestion, gluten/anti-gluten complexes are formed. The possible cross-reactivity between gluten and reticuli may be of great relevance here, for these soluble gluten/anti-gluten complexes could react with IgA ARA fixed on the dermal reticulin. Such a combination or the increased amount of IgA may alter the nature of the IgA present to a form which closely resembles, or acts, like, aggregated IgA, and subsequently activates the alternate pathway. Alternatively, IgA anti-gluten antibodies on their own, or circulating immune complexes, which are found in higher incidence in D.H. patients not on a gluten-free diet, (Mowbray et al 1973) may act in a similar manner in triggering off alternate pathway complement activation. A remote possibility is that gluten itself or a macromolecular complex of it, with no attached immunoglobulin, can, in its own right be the appropriate trigger. A corollary to this is the finding that other non-immunoglobulin substances like zymosan, lipopolysaccharide and bacterial endotoxin can activate the alternate pathway.

The possible mechanisms by which the skin lesions in D.H. are produced are summarised in Figure V.1. As to whether such mechanism(s) are responsible for the small-intestinal lesion in D.H. and coeliac disease remains to be seen. Doe et al (1973) have reported circulating immune

complexes in patients with coeliac disease. Shiner and Ballard (1972) described the presence of IgA and C3 in the lamina propria in the small bowel of children with coeliac disease following gluten challenge and suggest that the damage is an immunological and complement mediated one.

The absence of IgA in the skin in patients with coeliac disease alone is of great interest and suggests that there is a fundamental difference in the skin in these two patient groups. As to what this difference is, it is not possible to say at the moment. It might be that in D.H., there is a further abnormality - biochemical, immunological or otherwise - which facilitates IgA deposition, and arising from this, the predisposition to develop D.H. skin lesions. It may be that this fundamental abnormality is in the reticulin, which may be autoantigenic or modified biochemically to be immunogenic. Alternatively, the reported immune complexes in these two diseases may be of different physico-chemical makeup leading to different patterns of immune complex deposition and disease patterns. All these postulates, however, must be considered to be purely conjectural at the present time and need further study.

The question as to what the significance of ARA is also needs to be considered. This has already been extensively discussed and it would suffice here to summarize by saying that as a diagnostic aid, it is useful especially as a screening test in children. From an aetiological point of view however, the role of ARA remains uncertain. In D.H. it is possible that they are closely related to the pathogenesis of the D.H. lesion. The ARA can be of IgA class and in D.H. the incidence of circulating ARA is low. This low incidence however, may be due to increased deposition of the ARA (of IgA class) on the dermal reticulin. An experimental situation analogous to this occurs in autoimmune nephritis of sheep (Steblay, 1962). In these experiments, it was found that in sheep immunized with human placenta (HP), antibodies to HP could not be demonstrated in the circulation. Following nephrectomy however, such antibodies were found and it was suggested that all the circulating anti-HP antibodies were taken up by the kidneys in the

un-nephrectomised sheep. In D.H., the depositon of IgA ARA on the reticulin may act as a 'primer' for subsequent immunological events to take place, leading to the formation of the D.H. blister. For this to happen however, one will have to postulate a very fundamental abnormality in D.H. which allows or predisposes to this IgA deposition.

Taken together, the studies reported here indicate that immunological abnormalities are prominent in D.H. and coeliac disease and imply that they may be operative in the pathogenesis or perpetuation of these diseases. As to whether they are of aetiological significance, or represent secondary phenomena remains to be seen. The role of cell-mediated immunity has not been assessed in this thesis and obviously needs to be investigated.

At all events, the results here provide strong circumstantial evidence for a direct link between the gut and the skin in D.H. It is suggested that D.H. is a syndrome in which gluten is involved in the pathogenesis of both the skin and small intestinal lesions. In coeliac disease, this is confined only to the gut with sparing of the skin. As to why this should be so, no satisfactory answer can be given at the present time. In the context of this 'unitarian' concept, the rash of D.H. should be considered to represent 'gluten-sensitivity of the skin'.

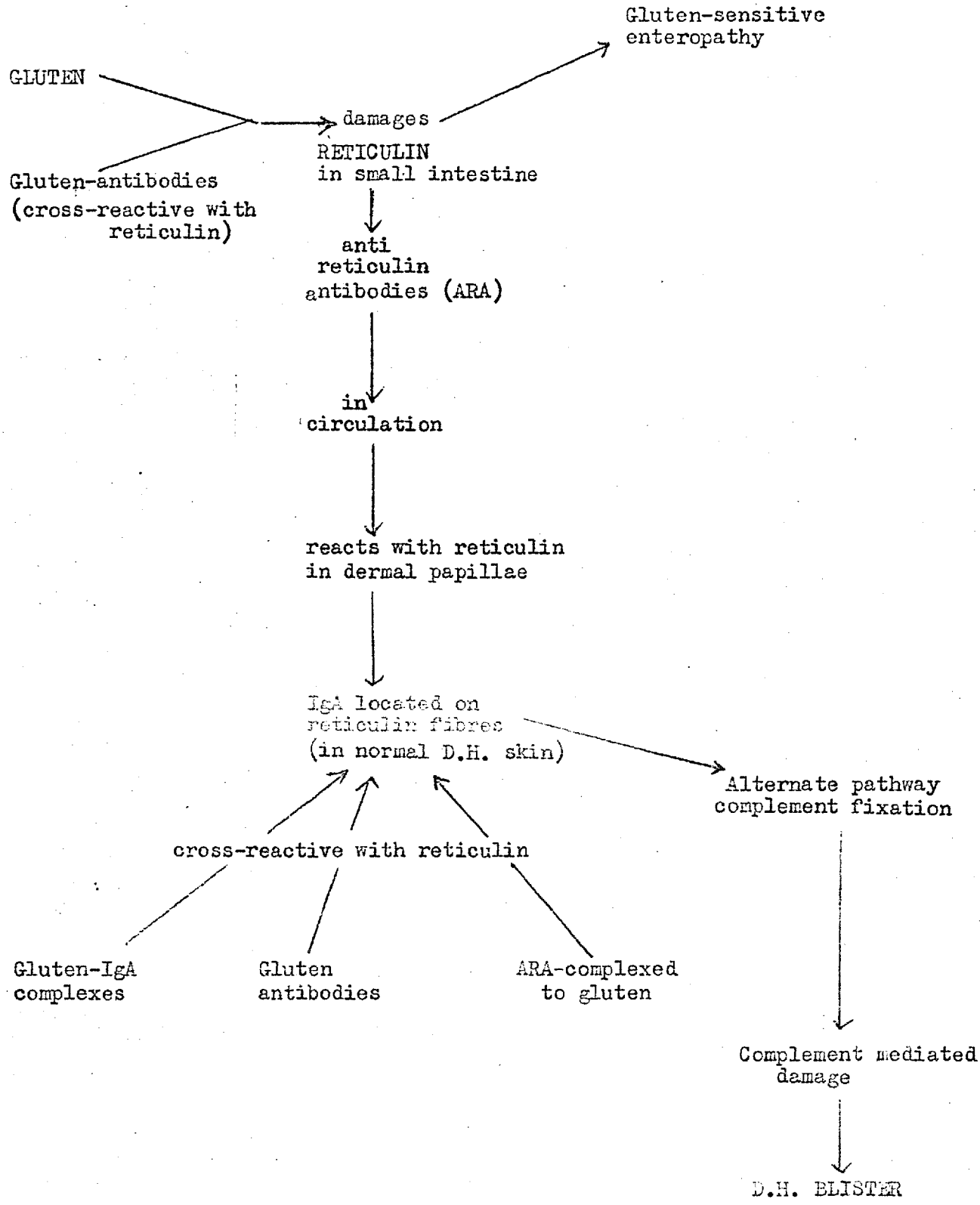


Figure V.1. - Possible mechanisms in the pathogenesis of the skin and small-intestinal lesions in dermatitis herpetiformis.

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TISSUE ANTIBODIES IN DERMATITIS HERPETIFORMIS AND ADULT CŒLIAC DISEASE

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Summary A new antibody of IgG class reacting with connective tissue in rat and human organs was found in 17% of 29 patients with dermatitis herpetiformis and in 36% of 31 patients with adult cœliac disease. The antibody appeared to be directed against reticulin rather than basement membrane. In 34% of the dermatitis-herpetiformis patients antinuclear factors of IgG or IgM class were present.

INTRODUCTION

DURING the past few years it has been shown that approximately two-thirds of patients with dermatitis herpetiformis (D.H.) have an enteropathy similar to that found in adult cœliac disease (A.C.D.) as judged by macroscopic and histological features of the small-intestinal mucosa.[1-3] This enteropathy improves in the majority of patients with D.H. on gluten withdrawal [4-7] and relapses on the reintroduction of gluten.[5] The skin lesions of D.H. have also been reported to improve with gluten withdrawal and relapse on the reintroduction of gluten,[5] and a direct relationship between the skin and small-intestinal disorder was suggested by Fry et al.[5] The cause of the skin lesions in D.H. and the enteropathy in D.H. and cœliac disease are as yet unknown. However, recent studies have suggested that there may be an immunological abnormality in these disorders as suggested by the following:

(*a*) Low serum-IgM levels in D.H.[3] and in A.C.D.[8]
(*b*) A decreased rate of synthesis of IgM in A.C.D.[9]

(*c*) The number of immunoglobulin-containing cells in the lamina propria in A.C.D. is greater than normal, and the proportion of IgM and IgG cells to IgA cells is increased.[10–12]

(*d*) IgA deposits have been found in the upper dermis below the basal membrane in D.H.[13,14]

(*e*) Antibodies reactive with the basement-membrane zone of jejunal mucosa but not of skin have been reported in D.H., and fixed γ-globulin has been demonstrated in the basement-membrane zone of the jejunal mucosa of 2 D.H. patients, in whose sera antibody could not be demonstrated.[15]

(*f*) Splenic atrophy in a proportion of patients with A.C.D.[16] and D.H.[3]

Because of these findings we have undertaken further immunological studies in D.H. and A.C.D., and report here on antibodies found in the sera of patients with these disorders.

PATIENTS AND METHODS

29 patients with D.H., 31 patients with A.C.D., and 28 healthy control subjects were studied. In the D.H. group there were 14 males with an age-range of 22 to 71 (average 43) years and 15 females whose ages ranged from 28 to 62 (average 41) years. In the A.C.D. group there were 7 males with an age-range of 21 to 56 (average 44) years and 24 females with an age-range of 16 to 74 (average 43) years. The control group comprised 13 males with an age-range of 20 to 79 (average 47) years and 15 females with an age-range of 19 to 53 (average 33) years.

One serum specimen from each patient or control subject was examined for antibodies to nuclei, smooth muscle, gastric parietal cells, mitochondria, and human thyroid microsomal antigen, using standard indirect immunofluorescent methods. The antigens were provided by cryostat sections of rat liver [17] (nuclei), stomach (parietal cells [18] and smooth muscle [19]), and kidney [20] (mitochondria), and human thyrotoxic thyroid [21] (thyroid microsomes). Thyroglobulin antibodies were detected by tanned-red-cell hæmagglutination,[22] and agglutinating factor against *Lactobacillus casei*, described by Cowan, Hoffbrand, and Mollin,[23] was also tested for by the method of Cowan.[24] The 88 sera were randomised and tested initially at 1/10 dilution; results were read without knowledge of the code. For the indirect immunofluorescence tests specific anti-IgG, anti-IgM, and in some batches anti-IgA conjugates (Burroughs Wellcome) were used, and specimens were examined on a 'Diapan' microscope with a 'Balzer' FITC-3 interference primary filter and a Shott and Genossen O.G.I. secondary filter.

Collagenase Treatment

Collagenase form II (Sigma) was dissolved at 1 mg. per ml.

PREVALENCE OF ANTINUCLEAR FACTOR AND CONNECTIVE-TISSUE ANTIBODIES

Group	Antinuclear factor		Antibodies to connective tissue				
	No. tested	Total pos.	No. tested	Positive			
				Total	a*	b*	c*
Dermatitis herpetiformis:							
No.	29	10	29	5	3	2	0
%	..	34·5	..	17	..	..	..
Adult cœliac disease:							
No.	31	2	31	11	2	7	2
%	..	6	..	36	..	..	..
Controls:							
No.	28	1	28	0	0	0	0
%	..	4	..	0	..	..	..

* Patterns of immunofluorescent staining: see text.

in 0·05*M* tris buffer containing 0·005*M* calcium chloride, pH 7·0, and unfixed cryostat sections on slides were covered with this solution and incubated at 37°C for from 1 to 18 hours. The slides were then washed in phosphate-buffered saline (pH 7·2) for 5 minutes and the sections treated with sera (1/5 dilution) and conjugates in the usual way. Control sections were treated with buffer only. The effect of the collagenase was demonstrated by staining control and enzyme-treated sections with van Gieson's stain for collagen and with silver stain (Gomori) for reticulin.

Periodate Treatment

Unfixed cryostat sections of rat liver, stomach, and kidney were pretreated with a 0·025*M* solution of sodium periodate ($NaIO_4$) at room temperature for periods of 15 seconds to 3 minutes; untreated sections, and sections pretreated with 0·025*M* solution of sodium iodate ($NaIO_3$) in parallel, acted as controls. The slides were then washed for 5 minutes in phosphate-buffered saline (pH 7·2) and treated with sera (1/10 dilution) and conjugates in the usual way.

Results

Antinuclear Factor (table)

10 (34·5%) of the 29 patients with dermatitis herpetiformis had serum antinuclear factor (A.N.F.) with titres ranging from 1/20 to 1/160; 6 were predominantly of the IgM class and 4 IgG. 1 in the control group (IgG, 1/10) and 2 in the cœliac group gave positive tests (1/20–1/40). This is a significant increase in D.H.

Antibodies against Connective Tissue (table)

In 5 (17%) of the D.H. sera and 11 (36%) of the

cœliac sera a new antibody of IgG class was found. With rat stomach, liver, and kidney sections, this produced staining of connective tissue in several different patterns, as shown in fig. 1. These patterns were:

(*a*) Staining of well-defined adventitial fibres around blood-vessels, of finer but also well-defined fibres in the liver sinusoids, and between the gastric glands and in the submucosa, and in the kidney of linear peritubular and peri-glomerular fibres, though not usually of the glomerular tuft itself.

(*b*) A more diffuse connective-tissue staining seen especially in the liver sinusoids and adventitia of blood-vessels, around and between the kidney tubules, often involving Bowman's capsule. Sometimes the glomerular tuft showed staining, but this was weaker in intensity. This pattern seemed to involve a fine network of connective-tissue fibres.

(*c*) Two of the cœliac sera showed an even more diffuse staining of connective tissue, of homogeneous appearance suggesting uptake by a component of ground-substance.

On repeated testing each positive serum always showed the same pattern (see accompanying table). Similar staining was seen with human fetal liver, small-gut, and skin sections. In the fetal small gut the lamina propria especially showed a fine network of stained fibres (fig. 2). Pretreatment of unfixed cryostat sections of the various tissues with purified collagenase did not abolish staining but rather enhanced its brightness with some of the positive sera. The activity of the collagenase was shown by the fact that, in sections treated in parallel, the characteristic staining with van Gieson was lost. Treatment of sections with periodate, however, abolished their ability to give the positive immunofluorescent patterns described.

Gastric Parietal-cell Antibody

This was not present in any of the control sera, but was found in 3 of the sera from patients with D.H. and 4 with adult cœliac disease.

Smooth-muscle Antibody

This was not found in the sera of controls, or of patients with D.H. or adult cœliac disease.

Thyroid Microsomal Antibody

This was found in 1 of the control sera, in 5 of the sera from patients with D.H., and in 3 of the sera from patients with adult cœliac disease.

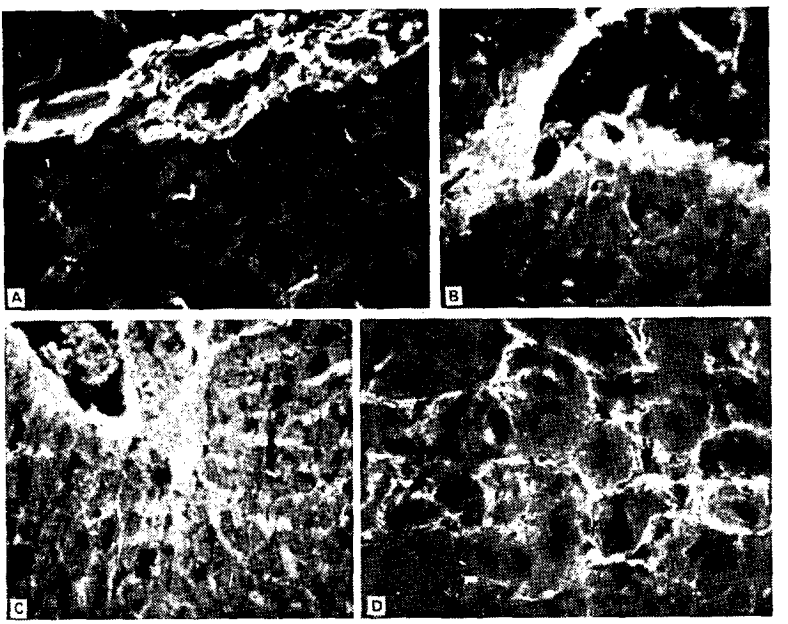

Fig. 1—Indirect (anti-IgG) immunofluorescent staining patterns on cryostat sections of tissues.

(Reduced by two-thirds from ×240.)

A, Rat liver. D.H. serum staining coarse fibres in adventitia of blood-vessels, and similar fibres sparsely distributed in the sinusoids.

B, Rat liver. D.H. serum staining finer fibres of similar distribution.

C, Rat liver. Cœliac serum staining fine network of fibres in adventitia and liver sinusoids.

D, Rat kidney. D.H. serum staining peritubular fibres, Bowman's capsule (top left), and adventitia (bottom right).

Thyroglobulin Antibody

This was not present in the sera of D.H. patients; there was 1 positive (titre 1/50) in a patient with adult cœliac disease.

Agglutinating Factor to L. casei

This factor was present in 16 of 26 sera from patients with D.H. and in 19 of 30 sera from patients with adult cœliac disease.

Mitochondrial Antibody

This was not present in the control sera, but was found in 1 of the D.H. sera and 2 of the sera from patients with adult cœliac disease.

Fig. 2—Human fetal (16-week) jejunum.
D.H. serum staining reticular network of lamina propria between two crypts.

Discussion

These results show that in patients with dermatitis herpetiformis the prevalence of A.N.F. is significantly greater than normal, and also that they may have antibodies against connective tissue. In patients with adult cœliac disease the prevalence of A.N.F. was normal, but the antibodies against connective tissue were found in an even higher proportion. These antibodies, giving the immunofluorescent staining patterns described, have not, to our knowledge, been observed previously. Autoantibodies against connective tissue have indeed been sought chiefly in connective-tissue diseases, but not usually found, although there are reports of anti-collagen antibodies,[25] and of antibodies against reticulin, in rheumatoid arthritis.[26] The anti-connective-tissue antibodies we have demonstrated in both cœliac disease and dermatitis herpetiformis are probably autoantibodies, since they react with human as well as rat tissues; but they do not seem to be directed against collagen, since pretreatment of tissue sections with collagenase did not impair, and even enhanced, the immunofluorescent staining. These patterns resemble very closely those produced by silver-impregnation staining techniques, which suggests that these autoantibodies may be directed against reticulin antigens, and the effects of periodate treatment on the reac-

tivity of the antigens involved supports this conclusion. Williamson et al.[26] and Loewi [27] have shown that anti-reticulin antibodies, like those described here, are neither organ-specific nor species-specific; and the fact that our antibodies react only weakly, or not at all, with renal glomeruli, while brightly staining connective-tissue fibres in Bowman's capsule and elsewhere, seems to accord with Scott's [28] inference from immunohistochemical analysis of connective-tissue antigens that some of the antigens of reticulin are distinct from those of renal glomerular basement-membranes.

The antibodies we describe here appear to be distinct from the tissue-antibodies reported in dermatitis herpetiformis by Dick et al.,[15] which react preferentially with human jejunal basement-membrane, especially from patients with dermatitis herpetiformis. The presence of these latter antibodies may well be related to basement-membrane damage, but this is a non-specific feature of many lesions involving epithelial structures. The finding of antireticulin antibodies in both the conditions studied here suggests that the fault in enteropathies of this sort may lie not, as has often been assumed, in the intestinal epithelial cells (nor in the basement-membrane which owes its origin to them [29,30]), but rather in the underlying connective tissue.

Antinuclear autoantibodies, in contrast, are found chiefly in patients with connective-tissue diseases, but occur also in patients with certain other disorders that are characterised by or associated with autoimmunity—e.g., chronic active hepatitis and myasthenia gravis. They are usually absent in organ-specific autoimmune conditions such as lymphocytic thyroiditis and pernicious anæmia. The true significance of serum antinuclear factors is not known, but the indications are that raised titres may reflect disturbance or impairment of immune function, and our findings therefore suggest that there may be a more general immunological disturbance in dermatitis herpetiformis than in cœliac disease.

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ANTI-RETICULIN ANTIBODIES IN CHILDHOOD CŒLIAC DISEASE

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Summary 19 patients with childhood cœliac disease and 28 controls have been investigated for the presence of anti-reticulin antibody. The antibody was found in 14 of the 19 patients with cœliac disease. 10 of these 19 were on a normal diet, and in 9 of these the antibody was found; whereas in the 9 patients on a gluten-free diet, it was found in 5. The antibody was also found in 1 of 28 control subjects. The anti-reticulin antibody is apparently specific for cœliac disease and may be of help in the diagnosis of the disease.

Introduction

A NEW antibody reacting with reticulin in rat and human organs has been described by us in a number of patients with dermatitis herpetiformis (D.H.) and adult cœliac disease (A.C.D.).[1] The significance of this antibody is unknown as it was not found in all patients with D.H. and A.C.D. In the past a distinction has frequently been made between cœliac disease occurring in children and in adults. We have therefore now undertaken a study to determine the presence and incidence of this antibody in childhood cœliac disease (C.C.D.) and its possible significance in the ætiology of this disorder.

Patients

The sera from 19 patients with C.C.D. were studied. Their ages ranged from 7 months to 14 years and there were 5 males and 14 females. The clinical diagnosis was confirmed by small-intestinal biopsy in all patients except 1, in whom the procedure failed for technical reasons. In this patient there was hæmatological evidence of cœliac disease and a good clinical response to a gluten-free diet (G.F.D.), supporting the diagnosis. At the time the serum was taken, 10 patients were on a normal diet and 9 were on a G.F.D.

In addition, sera from 28 children of similar sex and age distribution were also studied. These patients had other intestinal disease or were cases of " failure to thrive " who did not have cœliac disease as judged by the histological appearances of the small-intestinal biopsy.

Methods

One serum specimen from each of the 19 cases of C.C.D. and 28 control subjects were examined. These were collected, coded, and randomised by A. V. H. and M. A. R. and read independently without knowledge of the code by E. J. H. and P. P. S. The sera were examined initially at a dilution of 1/10 using standard indirect immunofluorescent methods. The antigens were provided by unfixed cryostat sections of rat liver, stomach, and kidney as described previously.[1] For the indirect immunofluorescence tests, specific anti-IgG, anti-IgM, and anti-IgA fluorosceinated conjugates (Burroughs Wellcome) were used at dilutions at which no non-specific staining was seen with normal sera. Specimens were examined on a Riechert 'Diapan' microscope with a 'Balzer' FITC-3 interference primary filter and a 'Wratten' 12 secondary filter.

Results

Childhood Cœliac Disease

14 (74%) of the 19 patients had anti-reticulin antibody of IgG class in their sera. In 9 of these 14 antibody of IgA class was also present. Antibodies of IgM class were not found. Of the 10 patients on a normal diet, 9 had the antibody in their serum, whilst in 9 patients on a G.F.D. the antibody was found in only 5.

Control Group

The anti-reticulin antibody was found in only 1 of the 28 in the control group (IgG class). This was a patient aged 1 year with diarrhœa and failure to thrive. The small-intestinal biopsy was reported as having a normal histological appearance.

Discussion

These results show that approximately three-quarters of the children with cœliac disease that we

examined have the anti-reticulin antibody in their sera. This is over four times the frequency reported in D.H. and twice the frequency in A.C.D. found by Seah et al.[1] It is well known that the small-intestinal lesion in D.H. is comparatively mild in the majority of patients [2-4] and clinically does not give rise to the symptoms and signs usually associated with cœliac disease. In fact, in the series reported by Fry et al.[2] no patient had symptoms or signs suggestive of cœliac disease. In cœliac disease presenting for the first time in adult life, it is generally assumed that the disorder has been present since childhood but has remained " latent ". This suggests that A.C.D. also is not so severe a disorder as that which presents with obvious clinical manifestations in childhood. Thus, the very high frequency of the anti-reticulin antibody in cœliac disease in children may well reflect the fact that the disorder is more severe in this group of patients, and therefore does not remain latent as in A.C.D. and D.H.

Our results also suggest that the antibody may disappear when patients respond to a G.F.D., as there was a lower frequency in the children on a G.F.D. than in those on a normal diet. In some patients with D.H. in whom this antibody has been found, it has been shown to disappear with gluten withdrawal and clinical improvement of the patient (Seah et al., unpublished). It is thus possible that the anti-reticulin antibody cross-reacts with gluten in some manner. In addition, the antibody does appear to reflect the sensitivity of the gut to gluten in cœliac disease as seen in its various clinical types: namely, C.C.D., A.C.D., and the most mild form as seen in D.H.

The results of this present study and our previous findings [1] suggest that the presence of the anti-reticulin antibody is specific for cœliac disease. The fact that it was found in one of our control subjects raises interesting possibilities since the child had clinical symptoms suggestive of C.C.D., and the fact that the small-intestinal biopsy was reported as normal as assessed by routine histology may not exclude the diagnosis. Thus, Fry et al.[2] reported patients with villi of normal appearances and columnar epithelium; yet there was gross cellular infiltration of the lamina propria, indicating small-intestinal disorder. In addition, Brow et al.[4] have shown that the enteropathy may be patchy and can only be detected by multiple biopsies. Thus, it is still possible that our control patient with the antibody may have cœliac disease,

and it could well be that the presence of the anti-reticulin antibody may be a new and helpful test in establishing the diagnosis of cœliac disease.

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IMMUNOGLOBULINS IN THE SKIN IN DERMATITIS HERPETIFORMIS AND CŒLIAC DISEASE

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Summary The unaffected skin of eighteen patients with dermatitis herpetiformis (D.H.), twenty-two patients with cœliac disease (C.D.), and eight controls were examined using direct immunofluorescence and class-specific fluorescein-conjugated anti-human IgA, IgM, and IgG antisera. All eighteen patients with D.H. showed IgA deposits in the skin: in seventeen the deposits were only found in the dermal papillæ; whilst in one it was found in a continuous line below the basement membrane, confirmed by immuno-electronmicroscopy. IgM deposits were also found in the dermal papillæ in three patients with D.H. and IgG deposits below the basement membrane in one patient. In cœliac disease, however, only one of the twenty-two patients showed papillary IgA deposits and one had continuous IgM deposits. These immunoglobulin deposits in D.H. and C.D. seem to be on the reticulin of the dermal papillæ. It is suggested that in D.H. there is a fault of the reticulin in the skin and small intestine, whilst in cœliac disease it is present in the small intestine but not in the skin. The reticulin cross-reacts with gluten complexes to give rise to an immunological reaction. In support of this hypothesis we have demonstrated cross-reactivity between gluten and reticulin.

Introduction

DERMATITIS herpetiformis (D.H.) is associated with

an enteropathy which is macroscopically and histologically indistinguishable from that found in cœliac disease (C.D.).[1–4] As in C.D. the enteropathy usually improves when the patient is put on a gluten-free diet,[5–7] and this diet has also been reported to improve the skin condition.[5,6] However, it has yet to be explained why patients with D.H. develop skin lesions and those with C.D. do not. Recent studies have shown a number of immunological abnormalities in both conditions. Low serum IgM-levels have been reported in both D.H. and C.D.,[3,8] although in C.D. the number of immunoglobulin-containing cells in the lamina propria of the small intestine is greater than normal and the proportion of IgM and IgG to IgA cells is increased.[9–11] In D.H., antibodies reactive with the basement membrane of the small intestine have been reported,[12] whilst antireticulin antibody has been found in the serum of patients with D.H. and C.D.[13,14] Also of note is the strikingly high frequency (34%) of antinuclear antibodies in D.H.[13] Immunoglobulin deposits have recently been described in the skin of D.H. patients.[15,16] We have studied, by direct immunofluorescence, skin from patients with D.H. or C.D. to see if there is any difference which may explain why skin lesions develop in D.H. but not in C.D.

Patients and Methods

Eighteen patients (six females, twelve males; age range 25–68) with D.H., twenty-two patients (fourteen females, eight males; age range 28–73) with adult C.D., and eight controls were studied. The criteria for diagnosis of D.H. were based on characteristic clinical features, histological changes, and response of the skin eruption to dapsone and relapse on withdrawal of the drug. All the cœliac patients had flat or flat-with-mosaic proximal small-bowel mucosa [17] and a characteristic cœliac mucosal lesion histologically.[18] The controls comprised four healthy volunteers and four inpatients with chronic bronchitis or cardiac ischæmia who consented to skin biopsy. Twelve of the eighteen D.H. patients were on a gluten-free diet and in seven the skin lesions were controlled by the diet alone. All the remaining patients were taking dapsone for control of their eruption. Of the twenty-two patients with C.D., ten were on a strict completely gluten-free diet.

Skin was taken by 3 mm. punch biopsy, under 2% lignocaine local anæsthesia, from the buttock. In patients with D.H., the skin was taken from an unaffected area well away from any lesions. The specimens were immediately embedded in 'OCT-Lab Tek' (Ames Company, Indiana), snap-frozen in precooled isopentane, and kept at −70°C until they were processed. 4 μ sections of the skin were

cut on a cryostat, transferred to glass slides, and air dried unfixed for 30 minutes. The sections were then washed in phosphate-buffered saline (P.B.S.) pH 7·2 for 15 minutes and subsequently treated with fluorescein-conjugated anti-immunoglobulin for 30 minutes. They were then given a final wash of 1 hour in P.B.S. and mounted in buffered glycerol (pH 8).

Burroughs Wellcome class-specific sheep-anti-human IgG, IgM, and IgA fluorescein-conjugated (F.I.T.C.) antisera were used in dilutions of 1/24, 1/24, and 1/12 respectively. The specificities of the conjugates were previously assessed by reacting them against smears of bone-marrow cells from cases of monoclonal myeloma [19] and shown to be class specific. Specimens were examined on a Reichert 'Diapan' microscope with a quartz-iodine light source, a Balzer F.I.T.C.-3 interference primary filter, and a Wratten 12 secondary filter.

Results

Dermatitis Herpetiformis

All eighteen patients showed immunoglobulin deposits in the skin, irrespective of whether the rash was controlled by a gluten-free diet or dapsone. Three patterns of immunofluorescent staining were seen (see table).

Papillary.—Immunofluorescent staining of the dermal papillæ was present in seventeen of the eighteen patients and was seen as a microgranular (fig. 1) or fibrillar (fig. 2) pattern as described by Chorzelski

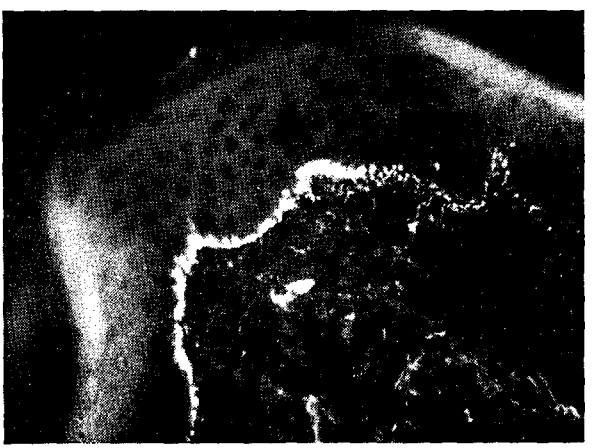

Fig. 1—D.H. skin: direct immunofluorescence using anti-IgA, showing microgranular papillary pattern, especially dense on the left.

(Reduced to ¾ of ×240.)

et al.[16] On close inspection it seemed likely that, in both cases, fibres are stained and the plane of section determined whether the appearance was fibrillar or microgranular. In seventeen patients positive staining was seen with anti-IgA and in three patients with anti-IgM also. No anti-IgG deposits were found.

Continuous.—Under low power with both anti-IgG and anti-IgA, one biopsy section showed a continuous line of immunofluorescence apparently delineating the basement membrane (fig. 3). Under higher power, however, the basement membrane itself was unstained and the immunofluorescence was located immediately below the basement membrane involving structures forming part of the dermal papilla. This was confirmed by immuno-electron-microscopy using horseradish-peroxidase-labelled anti-immunoglobulin (fig. 4).

Globular.—Specifically staining immunofluorescent globules (fig. 5) were found in the skin of six of the eighteen patients. This was seen with anti-IgA in four patients and anti-IgM in five. No anti-IgG deposits were seen. The globules were found in the upper dermis, at the dermo-epidermal junction, or in the basal layers of the epidermis itself. The situation of these globules could not be correlated with any known histological structure.

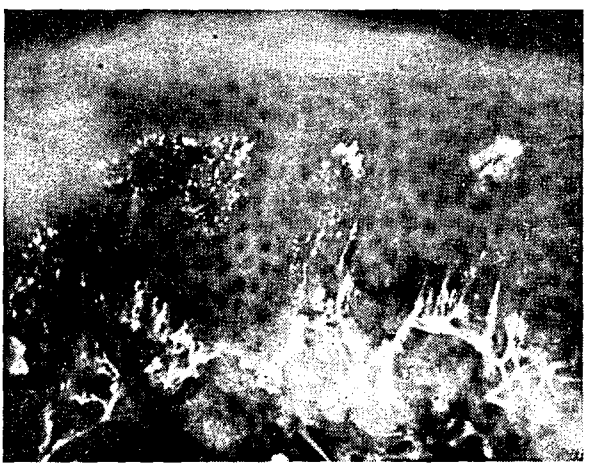

Fig. 2—D.H. skin: direct immunofluorescence using anti-IgA, showing predominantly fibrillar papillary pattern.

Areas of microgranular papillary immunofluorescence are also present (top left). (Reduced to ¾ of ×240.)

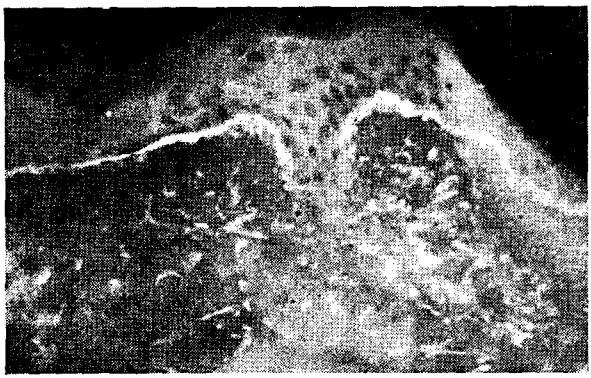

Fig. 3—D.H. skin: direct immunofluorescence using anti-IgA, showing continuous pattern.
(Reduced to ⅔ of ×240.)

Cœliac Disease

The findings were very different from those in D.H. Only one biopsy sample showed fibrillar papillary immunofluorescence with anti-IgA and one showed continuous immunofluorescence with anti-IgM. Globular deposits similar to those found in D.H. were present in fifteen of the twenty-two patients—eleven with anti-IgA and twelve with anti-IgM. No anti-IgG deposits were seen.

Controls

No papillary or continuous immunofluorescent patterns were present. However, in six of the eight specimens, anti-IgM globules were present. Anti-IgA globules were seen in two of these six. No anti-IgG deposits were seen.

Discussion

These results show a striking immunological difference between the unaffected skin in patients with D.H. and C.D., and have important implications. They confirm the reports of van der Meer [15] and Chorzelski et al.[16] that IgA deposits are present in the skin in D.H. In seventeen of the eighteen D.H. skin biopsies that we have examined, the fluorescence was localised to the dermal papillæ. On histological grounds, Pierard and Whimster [20] suggested that the earliest change in D.H. was in the dermal papillæ. The principal site of reticulin localisation in the skin is the dermal papillæ, and a proportion of patients

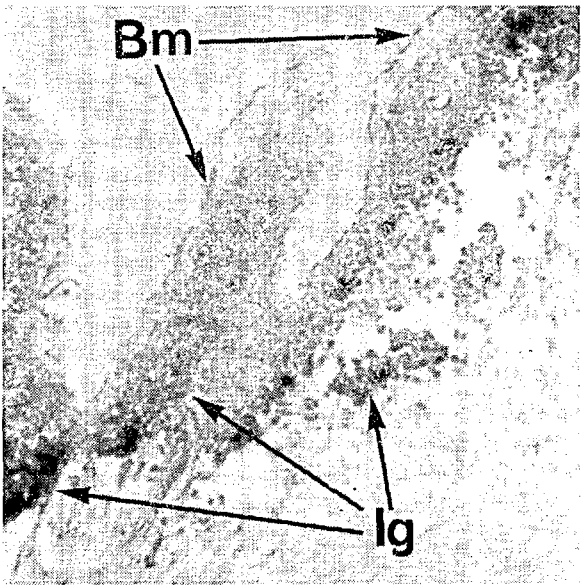

Fig. 4—D.H. skin: immuno-electronmicroscopy using horseradish-peroxidase-conjugated anti-IgG, showing immunoglobulin deposits (Ig) situated below uninvolved basement membrane (Bm).

(Reduced to ⅔ of ×19,000.)

with D.H. show antireticulin antibody in the serum. We suggest, therefore, that the immunoglobulin deposits in the skin in D.H. are on the reticulin fibres and that this is the primary site of the lesion. Immuno-

PATTERNS OF IMMUNOFLUORESCENCE

Group	No.	Immunopathology			
		Pattern of immuno-fluorescence	IgG	IgM	IgA
Dermatitis herpetiformis	18	Continuous	1	0	1
		Papillary	0	3	17
		Globular	0	5	4
Cœliac disease	22	Continuous	0	1	0
		Papillary	0	0	1
		Globular	0	12	11
Controls	8	Continuous	0	0	0
		Papillary	0	0	0
		Globular	0	6	2

Fig. 5—C.D. skin: direct immunofluorescence using anti-IgM showing globules.

(Reduced to $\frac{3}{4}$ of $\times 240$.)

electronmicroscopy in our 18th patient showed that the continuous staining pattern was beneath the basement membrane and, therefore, could also be reticulin.

Our findings establish that the primary sites of the lesion in D.H. and pemphigoid are different. In pemphigoid, immunoglobulins are bound to the basement membrane, whilst in D.H. they are fixed to the dermal reticulin. The frequency of antireticulin antibody in the sera of D.H. patients is rather low (17%),[13] but this might well be due to uptake of most of the available antibody by excess dermal reticulin antigen. An analogy occurs in autoimmune nephritis of sheep.[21] This explanation could be tested by eluting the immunoglobulin from skin samples from seronegative D.H. patients and testing this eluate for antireticulin activity.

Deposition of antireticulin antibodies in the skin could not by itself explain the disease process in D.H. because deposits of immunoglobulin are seen distributed in unaffected skin. An additional factor may be required before skin lesions develop in D.H., and a clue to the nature of this factor may come from the observation that a gluten-free diet improves the skin lesions.[5,6] It may be significant that the antireticulin antibody in two patients with D.H. was completely removed from the serum by absorption with gluten and gluten-fraction III (as prepared by Frazer et al.[22]) (Seah et al., unpublished). In these cases, therefore, the antireticulin antibody cross-reacted exten-

sively with a component of gluten. If soluble gluten/anti-gluten complexes were circulating in this patient and antireticulin antibodies cross-reacting with gluten were fixed in the dermal reticulin, then gluten complexes might be expected to be trapped in the skin. If anti-gluten antibody were IgG or IgM and complement fixing, this could explain previous reports of deposits of IgG, IgM, and complement, as well as IgA in the skin.[15] These speculations would receive support if gluten antibodies could be demonstrated in eluates from D.H. skin. Collections of eosinophils and polymorphs are prominent in the histological "microabscesses" characteristically found in the dermal papillæ, and these may be due to the chemotactic properties of complement locally activated by immune complexes. It seems possible that it is by preventing this end stage of the pathological process that dapsone exerts its therapeutic effect in suppressing the rash.

Immunoglobulin deposits were found in only two of the twenty-two cases of C.D. This suggests that by and large the skin in C.D. does not share the same immunological abnormalities of D.H. The two cases of C.D. with immunoglobulin deposits in the skin may well be latent D.H. (indeed one of these two patients subsequent to completion of these studies has been found to have an itchy rash on the elbows consistent with D.H.) and there are reports in the literature of patients with C.D. who subsequently develop D.H.[7,23] It may be that routine treatment of C.D. with a gluten-free diet suppresses clinical expression of latent D.H. We do not know what differences there may be in the dermal reticulin or the patients' immune response to dermal reticulin between D.H. and C.D., but the strong clinical and immunological overlap between the two diseases adds weight to our previous suggestion [13] that the primary site of damage in C.D., by analogy with our results in D.H., may be in the reticulin of the lamina propria of the small intestine. The finding of splenic atrophy in D.H.[24] as well as in C.D.[25] suggests that the immunological reactivity of reticulin may be widely disturbed in both disease states, primarily affecting the gut and lymphoreticular system in C.D., but in D.H. the skin as well as these other two tissues. Whether reticulin is abnormal in these conditions or is an "innocent bystander" of antibodies produced in response to gluten remains uncertain.

Finally, the fluorescent globular deposits found in

many of the biopsy specimens deserve mention. These stained specifically with anti-IgA and/or anti-IgM but not with anti-IgG conjugates, and occurred as often in normal skin as in the test samples. They seemed to be simply small extravascular deposits, not associated with cellular or other histological structures and playing no pathological role. They perhaps represent a physiological process of immunoglobulin transudation in the skin.

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CLEARANCE OF SKIN LESIONS IN DERMATITIS HERPETIFORMIS AFTER GLUTEN WITHDRAWAL

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Summary 24 patients with dermatitis herpetiformis (D.H.) have been treated with a gluten-free diet (G.F.D.) for periods varying from four months to five years. Of 20 patients who have taken a G.F.D. for a year or longer, 16 (80%) have been able to stop taking dapsone or substantially reduce the dose of the drug. 10 patients have been able to stop taking dapsone completely and are now free of all skin lesions. No patient was able to reduce his dapsone requirements before five months, and 1 patient took twelve months to do this. The length of time taken to be able to stop dapsone completely varied from eight to forty-eight months. In another group of 20 patients with D.H. who did not take a G.F.D. but have been followed up for a similar length of time, there was no significant alteration in dapsone requirements. Reintroduction of gluten in 4 patients who were completely free of skin lesions on a G.F.D. produced irritation and blisters within a week in 3 and within three weeks in the fourth. Dapsone was required for the immediate control of these lesions, which were subsequently controlled again by a G.F.D. alone. Electron-microscopic studies have shown subepidermal membrane-bound vacuoles in clinically normal skin in all 7 patients studied whose eruption was being controlled by dapsone, but these vacuoles were not present in any of the 6 patients whose eruption was controlled by a G.F.D. alone. These results show that the skin lesion in D.H., like the gut lesion, is gluten dependent and that both lesions are part of the same disease process. The length of time for patients to become free of skin lesions after beginning a G.F.D. is stressed, and probably accounts for the previous reports in which it has been stated that the skin lesions are not influenced by a G.F.D.

Introduction

THE association of a cœliac lesion of the small intestine with the skin rash of dermatitis herpetiformis (D.H.) is well established. The intestinal lesion in D.H. resembles that of cœliac disease in responding to gluten withdrawal and relapsing on reintroduction of gluten.[1–4] But are the skin and gut lesions in D.H. part of the same disease process? The most widely held view is that the two lesions are of different ætiology and that the skin lesions are not related to gluten sensitivity.[3–10] We believe that this view is incorrect and arises because trials of a gluten-free diet (G.F.D.) on the skin lesions in patients with D.H. have been inadequate.

We have found that a strict, long-term G.F.D. benefits the skin lesions in almost all patients with D.H. In addition, following the observation that membrane-bound vacuoles are present in the clinically normal skin of patients with D.H. taking dapsone,[11] we have shown that these vacuoles are not present in patients whose eruption is controlled by a G.F.D. Taken together, these clinical and electron-microscope studies clearly show that the skin and intestinal lesions of D.H. are part of a single disease.

Patients and Methods

44 patients with D.H. have been studied. There were 22 males and 22 females. Their ages are 26–76 years. The diagnosis of D.H. was made on the characteristic clinical, histological, immunological (IgA deposits in the dermal papillæ of clinically normal skin) features, and the clearance of the eruption with dapsone and/or sulphonamides, and recurrence of the rash on withdrawal of the drug.

After full intestinal and hæmatological investigations, all patients had the nature of their illness explained to them and were offered treatment with a G.F.D. 35 of the 44 patients were willing to try this. The supervision of the diet and follow-up were carried out by one physician (L. F.) and the help of dietitians was enlisted when necessary. Patients were seen every six weeks for six months and then every three months. Before starting a G.F.D., all patients were instructed to reduce their dose of dapsone to the lowest level which completely controlled their skin lesions. After three months on a G.F.D., 11 of the 35 patients said they were unable to persist with it. Of the 24 patients who have persisted with the diet, 3 have admitted to occasionally taking food which they knew contained gluten. Another patient does not keep strictly to the diet.

At all consultations patients have been instructed to try and reduce their dapsone or sulphonamide drugs gradually, but at no time were they asked to stop suddenly. This has

applied equally to all patients whether or not they were taking a G.F.D. If the patients began to have irritation or skin lesions, they were instructed to increase the drug to the original dose and not to attempt further reduction of dosage for another month.

In 4 patients who were completely free of all skin lesions on a G.F.D., gluten was reintroduced to the diet for a period of three months.

Electron-microscopic Studies

Samples of clinically normal skin were taken from 6 patients whose skin lesions were controlled by a G.F.D. alone and from 7 patients taking dapsone only. The specimens were fixed in 4% glutaraldehyde and post-fixed in osmium tetroxide. Thin sections were cut, stained with uranyl acetate and lead citrate, and examined on a Siemens ' Elmskop I ' microscope.

Results

Group I: Rash Controlled by G.F.D. Alone

10 patients taking a G.F.D. have stopped their dapsone or sulphonamides completely and remain free of irritation and skin lesions (table I). No patient

TABLE I—PATIENTS WHOSE RASH IS CONTROLLED BY G.F.D. ALONE

Patient no.	Age	Sex	Duration of D.H. (yr.)	Original dose of dapsone (mg./day)	Time to reduce dapsone (mo.)	Time to completely stop dapsone (mo.)	Length of time on G.F.D. (yr.)
1	56	M	14	150	6	12	5
2	54	M	12	50	5	11	5
3	29	M	8	50	11	22	5
4	38	M	8	50	5	8	5
5	70	M	52	150	12	48	5
6	55	F	20	500*	5	13	4
7	31	F	14	100	6	12	4
8	62	F	21	50 mg. twice weekly	5	8	2
9	38	M	11	50	5	14	2
10	38	F	4	100	6	22	2

* Sulphamethoxypyridazine.

managed to reduce the dose of his drug before five months on a G.F.D. 8 of the 10 patients managed a slight reduction in their drug intake by six months. In the other 2 patients it was eleven and twelve months before the dose of dapsone could be reduced. The time taken to stop the drugs completely and remain free of skin lesions varied from eight to forty-eight months. After two to forty-eight months' follow-up all patients have remained free of skin symptoms and signs,

whilst adhering to their G.F.D. The duration of the skin disorder in these 10 patients ranged from four to fifty-two years, and at no time before the G.F.D. had any of them been free of skin lesions without taking drugs.

In 4 patients who had been free of all skin lesions for at least six weeks, gluten was reintroduced to the diet. 3 of the 4 patients developed irritation and blisters within a week and the fourth patient within three weeks. Dapsone was then required to control the eruption in the same dosage as before the G.F.D. After three months, a G.F.D. was re-established. All 4 patients again managed to control the skin lesions without dapsone, but the time taken to do this was virtually identical to the time taken when first taking a G.F.D.

TABLE II—PATIENTS TAKING A G.F.D. BUT ALSO REQUIRING DRUGS

Patient no.	Age	Sex	Duration of D.H. (yr.)	Original dose of dapsone (mg./day)	Length of time on G.F.D.	Present dose of dapsone (mg.)
1	47	M	6	100	4 yr.	50 every 5 days
2*†	35	M	8	150	5 mo.	50/day
3	46	F	4	100	3 yr.	50 twice weekly
4	52	M	27	100	1 yr.	50 every 3 days
5*†	36	F	6	200	2 yr.	100/day
6*†	35	M	3	200	21 mo.	100/day
7	49	F	26	100	4 yr.	100/day
8	63	M	7	100	5 yr.	100/day
9	69	M	6	50	3 yr.	50/day
10*	57	M	17	200	2 yr.	200/day
11	49	M	16	200	8 mo.	200/day
12	51	M	2	50	6 mo.	50/day
13	38	F	2	100	5 mo.	50/day
14	42	M	10	50 (alt. days)	4 mo.	50 (alt. days)

* G.F.D. is not strict.
† Has knowingly taken occasional gluten.

Group II: Rash Controlled by G.F.D. and Drugs

14 patients taking a G.F.D. also required dapsone to stop irritation and remain clear of skin lesions (table II). However, 6 of the 14 patients required significantly less dapsone than before the diet. All these 6 patients had been on a G.F.D. for more than a year, but 3 admitted to occasionally taking gluten. 3 (nos. 7, 8, and 9) of the 14 patients who had been on the G.F.D. for longer than three years failed to reduce their dose of dapsone. It was felt that these 3 patients adhered to a strict G.F.D. In a further patient (no. 10) who had

been on a G.F.D. for two years and showed no reduction of his dapsone requirements, it was apparent that his diet was not strictly gluten-free. Of the 4 remaining patients who showed no reduction in their dapsone requirements, all had been on a G.F.D. for less than eight months, for six months or less in 3 of the 4.

Group III: Rash Controlled by Drugs Alone

20 patients were not willing or able to take a G.F.D.

TABLE III—PATIENTS NOT ON A G.F.D.

Patient no.	Age (yr.)	Sex	Duration of D.H. (yr.)	Original dose of dapsone (mg./day)	Length of follow-up (yr.)	Present dose of dapsone (mg./day)
1	48	F	18	50	5	50
2	70	M	5	100	5	100
3	66	F	13	50	5	50
4	30	F	13	100	5	100
5	34	F	12	150	5	200
6	42	M	6	100 mg. (twice weekly)	5	100 mg. twice weekly
7	54	F	8	*	5	*
8	68	F	7	50	5	50
9	48	F	15	200	4	200
10	39	F	15	100	3	100
11	26	F	4	50	3	50
12	37	F	4	100	3	200
13	22	M	3	100	2	100
14	74	M	5	50	2	50
15	48	F	3	100 mg. (alt. days)	2	100 mg. (alt. days)
16	46	M	18	200	2	200
17	76	M	15	100	1	100
18	68	F	4	50	1	50
19	26	F	2	50 mg. (alt. days)	1	50 mg. (alt. days)
20	59	M	6	100	1	100

* Sulphapyridine 3 g. per day.

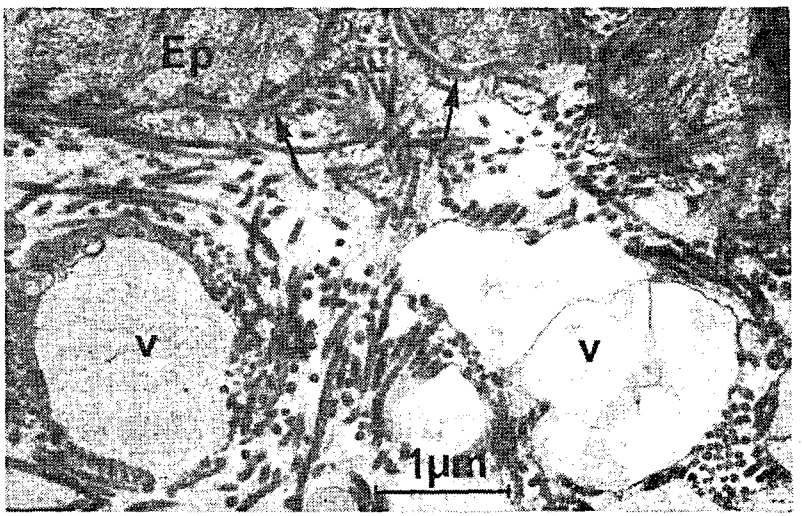
None of them could reduce the dose of dapsone after follow-up for over a year in all 20 and as long as two to five years in 16 (table III).

Electron-microscopic Studies

Membrane-bound vacuoles were found in the dermis below the basal lamina (figure) in the clinically normal skin from all 7 patients receiving a normal diet and taking dapsone. In contrast, no abnormality was seen in the skin of all 6 patients whose skin disorder was completely controlled by a G.F.D.

Discussion

These results show that the skin lesions in most patients with D.H. are gluten-dependent. 80% of



Membrane-bound vacuoles (V) are seen in the dermis just below the basal lamina (arrows) in clinically normal skin from a patient with D.H. solely on dapsone.
Ep = epidermis.

patients who took a G.F.D. for more than a year were able to stop or significantly reduce the dose of dapsone or sulphonamides. However, it took a long time on a G.F.D. before the patients could reduce the dose of their drugs and ultimately to stop these, and the G.F.D. had to be strict if the skin lesions were to improve substantially.

These findings amplify those based on a smaller group of patients with shorter periods of follow-up.[1,2] It is remarkable that, though the association between D.H. and the typical clinical, histological, and hæmatological features of adult cœliac disease was described six years ago,[12,13] and the improvement of the skin lesions with gluten withdrawal was reported soon afterwards,[1,2,14] it is still widely considered that the skin lesions are unrelated to gluten ingestion.[3,4,6,8,9] There appear to be at least four reasons for this continued misconception. First, as we found, improvement of the skin on gluten withdrawal takes many months or even years to manifest, and the two widely cited reports [3,4] suggesting no benefit from gluten withdrawal were based on a short follow-up period in small numbers of patients—six to nine months in 4 patients [3] and two to six months in 6 patients.[4] As shown here, this is not long enough to obtain clearance of the skin

lesions. Second, the G.F.D. must be strict. 3 of the 6 patients who reduced their dapsone requirements but could not stop the drug completely admitted to occasional gluten ingestion. These 3 patients themselves noted that their skin lesions were worse after gluten ingestion and then required more dapsone to control their eruption. Moreover, all 4 patients in whom gluten was reintroduced to the diet after their skin lesions had been completely controlled by a G.F.D. developed skin lesions which were eventually controlled again by gluten withdrawal. In the single patient reported by Shuster et al.[3] who had taken G.F.D. for seven years and still had skin lesions, adherence to diet may not have been strict because the morphological appearance of the small intestinal mucosa after seven years was still " flat ". These two features (i.e., length of time and strictness of the effect of a G.F.D. on the skin lesions in D.H.) are comparable to the effect of gluten withdrawal on the gut lesion in adult cœliac disease, since this may also take many months to revert to normal and is benefited little or not at all by only partial withdrawal of gluten.[15] Third, Marks and Shuster[5] suggested that during the natural history of D.H. requirements for dapsone vary widely from time to time and, thus, apparent benefit of the skin lesions from gluten withdrawal may, in fact, be no more than a spontaneous remission of the disease. In this respect, our control group of 20 patients who, for one reason or another, were not treated by gluten withdrawal show clearly that dapsone requirements in D.H. are remarkably constant over many years of follow-up. None of these patients could reduce their dapsone dosage. None of our patients treated with a G.F.D., whose length of histories of D.H. varied from four to fifty-two years, ever experienced a natural remission. Spontaneous remission of D.H., in our experience, does not occur, and before it can be claimed the original diagnosis must be checked on strict criteria. Finally, it must be remembered that not all patients with cœliac disease respond to a G.F.D.[16] It is, therefore, possible that a proportion of patients with D.H. also do not respond solely to a G.F.D. This may be the explanation for the 3 patients (7, 8, and 9) in our series not responding to a G.F.D. There has been a suggestion that in some patients with D.H. there is milk sensitivity as well as gluten sensitivity, and removal of milk from the diet is necessary for control of the skin lesions.[17]

One reason which has delayed general acceptance that the gut and skin lesions in D.H. are both due to gluten sensitivity is that dapsone benefits the skin lesion but not that of the gut. The electron-microscopic findings reported here together with those of Riches et al.[11,18] may help to explain the actions of both dapsone and gluten on the skin lesions. In patients whose eruption was completely controlled by dapsone, subepidermal vacuoles were present. When the dose of dapsone is reduced or withdrawn these vacuoles enlarge and seem to coalesce.[18] From this evidence it seems that dapsone does not stop the formation of the vacuoles but prevents them increasing in size to form a clinical blister. This observation would account for the sudden appearance of the rash when the drug is stopped. However, the initial lesions in the skin do seem to be gluten induced, since in our patients whose eruption was controlled completely by a G.F.D. no vacuoles were found. The exact explanation why dapsone does not benefit the gut is uncertain, though it may be related to differences in the way the two tissues, skin and gut, respond to a similar insult (gluten ingestion) and to differences in the way the immune system, activated by gluten, damages organs.

Taken together, the clinical and morphological results reported here do clarify the relation of D.H. and cœliac disease in showing that they are not, as widely thought, two distinct but genetically related diseases but rather that the syndromes of D.H. and cœliac disease are part of a single disease process in which gluten sensitivity plays a central role. This is crucial both in the clinical management of D.H. and to any concept of the pathogenesis of both cœliac disease and D.H.

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CIRCULATING IMMUNE COMPLEXES IN DERMATITIS HERPETIFORMIS

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Summary Immune complexes have been detected by an anti-complementary method in the sera of 12 (80%) of 15 patients with dermatitis herpetiformis (D.H.) taking a normal diet, and in 4 (36%) out of 11 taking a gluten-free diet. The size of the immune complexes was between 8 and 10*S*. Immune complexes were detected in the sera of only 3 (12%) of 25 D.H. patients by the C'1q method, but this method detected complexes in 9 (28%) of 32 patients with Crohn's disease, 2 of 6 patients with cœliac disease, and 2 of 6 patients with ulcerative colitis.

Introduction

GLUTEN plays a central role in the pathogenesis of cœliac disease (C.D.) and dermatitis herpetiformis (D.H.), but the precise mechanism by which gluten damages the small intestine in C.D. and D.H. and causes the skin lesions in D.H. is unknown. There is accumulating evidence, however, that immunological processes are involved. Low IgM levels [1,2] and antireticulin antibody [3] have been found in the sera of about a third of the patients. There is a proportional increase in IgM and IgG producing plasma-cells compared with IgA cells in the small intestinal mucosa in C.D.[4-6] In addition, antibodies reactive with the basement membrane of the intestinal epithelium have been reported,[7] and Shiner and Ballard [8] have demonstrated the presence of IgA and complement in the jejunal

mucosa after gluten challenge. Although gluten could damage the small intestine by direct contact, it could damage the skin only if a toxic fraction entered the bloodstream. However, IgA deposits occur in the dermal papillæ of the clinically unaffected skin in D.H.,[9–11] and fibrin [12] and complement [9,13] are present in the involved skin. On the basis of these observations, it seems possible that immune complexes may be formed in the intestine in response to gluten and circulate to the skin in D.H. to cause damage. We now report the presence of complexes in the sera of most D.H. patients taking a normal diet; these complexes are probably IgG, 8–10*S*, and complement fixing. We have compared the frequency of the complexes in D.H. with that in two other intestinal diseases—Crohn's disease and ulcerative colitis—in which skin lesions may also occur and in which immune mechanisms have also been suggested to play a role.

Patients and Methods

Patients

The sera from 26 patients with D.H., 32 with Crohn's disease, 6 with cœliac disease, and 6 with ulcerative colitis were examined. Of the 26 patients with D.H., 15 were taking a normal diet and 11 a gluten-free diet (G.F.D.). The tests were also carried out on the sera of 20 male and 10 female healthy adult volunteers.

Methods for Detection of Immune Complexes

Anti-complementary method.—The method is based on the binding of C'1 to circulating complexes after heat inactivation at 56°C of the C'1 which may have been bound in the blood, with a subsequent back titration of added guineapig complement. Fresh sera were stored at −70°C until assayed. They were thawed and heated at 56°C for sixty minutes in 0·1 ml. volumes. Guineapig complement (2·5 units) was added and the samples maintained at 4°C for thirty minutes. 0·1 ml. of a 3% suspension of optimally sensitised sheep red blood-cells were added and incubated at 37°C for a further fifteen minutes. The samples were then diluted with 1·0 ml. of barbitone-buffered saline (pH 7·2) and after centrifugation at 3000 *g* for five minutes the lysis was calculated from the released hæmoglobin in the supernatant using a Gilford 300N spectrophotometer. The curve for the complement titration was constructed using known standards, and the loss of hæmolytic complement activity was determined. The results are expressed as the units of complement left in the assay which had a lower 95% confidence limit of 1·1 units remaining.

C'1q method.—The method of Agnello et al.[14] was used with the gel buffer of molarity 10 m*M*.

Size of Circulating Immune Complexes

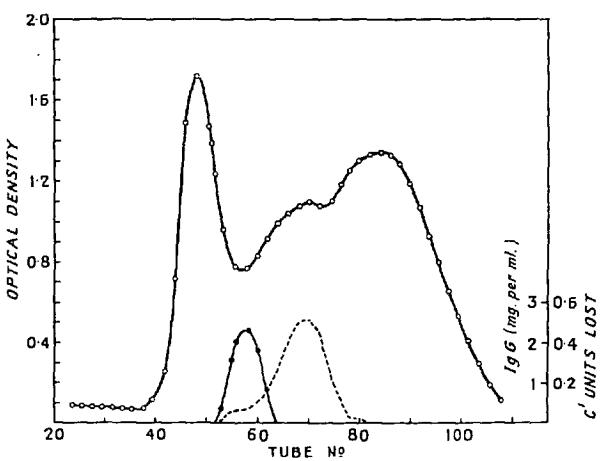
Using the anti-complementary technique described above, complexes in the fractions obtained by gel filtration of serum samples on columns of ' Sephadex G-200 ' could be assayed. The position of the activity was determined as were the positions of IgG, IgM, and IgA by radial immunodiffusion assay on the same samples. The isolated peak of activity was collected and applied to the top of a 5–44% sucrose density gradient in phosphate-buffered 0·15*M* saline, pH 7. The gradient was centrifuged at 410,000 *g* for 7 hours and the anti-complementary activity of the fractions from the gradient tubes determined and the density measured. Dialysis before assay was not required because sucrose, at concentrations below 30%, did not interfere with the assay. The molecular size of the complexes was determined by the gel-filtration technique using markers of known molecular weights, and their density was determined by the ultracentrifugation method.

Results

Dermatitis Herpetiformis

Anti-complementary method.—Circulating immune complexes were detected in the sera of 12 (80%) of the 15 patients taking a normal diet, but in only 4 (36%) of the 11 sera from patients taking a G.F.D.

C'1q method.—3 (12%) of the 25 patients showed immune complexes in the sera by this test. All 3



Chromatography of serum on sephadex G-200 from one patient, showing position of anti-complementary complexes.

●———● = Anti-complementary assay.
○———○ = Whole serum at O.D.$_{280}$.
- - - - - - - - = IgG.

O.D.$_{280}$ = Optical density at 280 nm.

positive results occurred in the sera of the 15 patients taking a normal diet.

Controls

None of the 30 controls gave positive tests by either the anti-complementary or the C'1q techniques.

Other Conditions

9 (28%) of 32 sera from patients with Crohn's disease showed the presence of immune complexes by the C'1q method, 2 of 6 sera from patients with cœliac disease, and 2 of 6 sera from patients with ulcerative colitis also showed the presence of immune complexes by the C'1q method. These sera were not tested by the anti-complementary method.

Size of Circulating Immune Complexes in D.H.

The size of the immune complexes detected by the anti-complementary method in 4 patients tested was found to be between 8 and 10*S* (see figure). This is the size of IgG complexes. In the four sera studied the densities of the complexes were found to be 1·22, 1·27, 1·41, and 1·37. All four figures are less than the density of nucleic acids and implies that these complexes are unlikely to be virus complexes.

Discussion

These results show that immune complexes are present in the sera of most patients with gluten-sensitive enteropathy and the skin lesions of D.H. The size of the complexes are between 8 and 10*S*, which implies that they are IgG complexes. We are uncertain why we detected complexes more often by the anti-complementary method compared with the C'1q method, but it is presumably related to differences in the sensitivity of the two methods or to differences in the exact type of complex which each technique detects.

Whether the complexes are involved in the pathogenesis of the skin and gut lesions or are secondary to this is at the present uncertain. The significantly lower frequency of circulating complexes in patients taking a G.F.D. could imply that these complexes contain part of the gluten molecule. The fact that these were present at all in four patients taking a G.F.D. may then be that the diet was not strictly gluten-free. Alternatively, it may be that the complexes do not contain part of the gluten molecule and it is possible that they are related to part of the reticulin molecule.

Anti-reticulin antibody occurs in D.H. and C.D.[3] The titre of anti-reticulin antibody falls slowly when the patient takes a G.F.D., and this shows a good correlation to the clinical results of the healing of the small bowel and skin. In addition, cross-reactivity has been demonstrated between fraction III of gluten and reticulin.[11] It may be, therefore, that if connective tissue is damaged by gluten, complexes might be formed for some considerable time later from this damaged connective tissue if this takes a long time to heal.

The finding of circulating immune complexes and the supposition that they migrate to the skin and cause lesions there would be compatible with the presence of complement [9,13] and fibrin [12] in the skin lesions. However, the fact that the upper part of the small intestine is more severely affected than the ileum would suggest that, rather than the damage to the gut being caused solely by circulating complexes, some form of direct effect of the gluten on the intestine might be operative.

Circulating immune complexes in Crohn's disease and ulcerative colitis were mentioned in an abstract.[15] Their incidence, however, was not recorded. We have found that almost a third of patients with each of these diseases have complexes in their sera by the C'1q method, and this is similar to that found by the anti-complementary method (unpublished).

However, before attributing a direct causative role to immune complexes in D.H., Crohn's disease, and ulcerative colitis, the possibility that these complexes are secondary to the disease process must be considered. Further studies are needed to define the characteristics of these complexes and to elucidate their role, if any, in the pathogenesis of the gut and skin lesions in these diseases.

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ALTERNATE-PATHWAY COMPLEMENT FIXATION BY IgA IN THE SKIN IN DERMATITIS HERPETIFORMIS

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Summary 34 samples of skin from nineteen patients with dermatitis herpetiformis (D.H.) were examined for the presence of IgA, IgG, and IgM and the C1q and C3 components of complement. IgA was present in all specimens but IgM was present in only 9 and IgG in 3. C3 was found in 16 (47%) of the specimens. The highest frequency was in the subgroup of specimens taken from early lesions (8 out of 9). However, C3 was also found in the 3 of 9 specimens of uninvolved skin from patients not receiving treatment, in 2 of 8 specimens from patients whose rash was controlled by dapsone, and in three of eight patients whose rash was controlled by a gluten-free diet. C1q was present in only 3 (9%) of the 34 samples; in 1 IgG was present and in 2 IgM. These results suggest that IgA activates complement by the alternate pathway in D.H. and that dapsone does not block this process.

Introduction

THE skin lesions in dermatitis herpetiformis (D.H.) are almost certainly due to an immunological disturbance. Immunoglobulin deposits, first found in the uninvolved skin in D.H. by Cormane,[1] are found in all patients with this disorder; IgA is always

present, and IgG and IgM are found occasionally.[2] The C3 component of complement has also been demonstrated.[3–5]

It is generally accepted that IgA does not bind complement via the "classical pathway" of complement activation, so the relevance of the findings of IgA and C3 in the skin have been unexplained. Our findings suggest that IgA activates complement by the "alternate pathway" in the skin in D.H., and that this mechanism of complement activation is involved in the disease process.

Methods

Biopsy

34 samples of skin from nineteen patients with D.H. were examined for C3 and C1q components of complement and for IgA, IgM, and IgG. The diagnosis of D.H. was made on the clinical picture, the response of the rash to dapsone, its relapse on withdrawal of therapy, and the presence of IgA in the uninvolved skin.[6] Biopsies were done under 2% lignocaine local anæsthesia with a punch or by excision from the uninvolved and involved skin from patients in various stages of treatment (table), and immediately embedded and oriented in 'OCT-Lab Tek' (Ames, Indiana) and snap-frozen in precooled isopentane. Specimens were stored at −70°C until processed.

Immunofluorescent Studies

Specimens were examined by direct (for IgG, IgM, IgA, and C3 deposits) and indirect (for C1q and IgA-secretory piece) immunofluorescence. The skin sections were processed and examined as previously described.[2]

Antisera and Conjugates

Anti-C1q.—This was prepared by immunising rabbits with C1q prepared from fresh human serum,[7] and was subsequently absorbed with pseudoglobulin. Immuno-

C3 DEPOSITS IN 34 BIOPSIES FROM 19 PATIENTS AND RELATIONSHIP TO C1q AND IgA, IgG, AND IgM DEPOSITS

Specimens	Total	IgA	IgG	IgM	C3	C1q
No treatment:						
Involved skin	9	9	0	1	8	0
Uninvolved skin	9	9	0	1	3	0
Rash controlled by dapsone	8	8	1	2	2	2
Rash controlled by G.F.D. alone	8	8	2	5	3	1
Total	34	34	3	9	16	3

diffusion studies gave a single precipitin line against both C1q and fresh human serum. The absorbed unconjugated C1q antiserum was used at a dilution of 1/5 throughout the study. A goat-anti-rabbit gammaglobulin fluoroscein (F.I.T.C.) conjugate (Nordic) was used as the second layer in a dilution of 1/10 in the indirect immunofluorescence studies.

Anti-C3.—A goat-anti-human C3 (β1c/β1a) F.I.T.C.-conjugated antiserum (Hylands) was used for detection of C3 deposits in the skin at a dilution of 1/5. The specificity of this conjugate was assessed by testing its ability to detect complement fixed by human IgG gastric/parietal-cell antibody.

Anti IgG, IgM, and IgA.—F.I.T.C.-conjugated sheep-anti-human IgG, IgM, and IgA (Wellcome Reagents) in dilutions of 1/24, 1/12, and 1/10 were used for detection of IgG, IgM, and IgA deposits in skin.[2] The specificities of the conjugates were previously tested by gel diffusion and by reacting them with monoclonal myeloma bone-marrow smears according to the method of Hijmans et al.,[8] and each was shown to be heavy-chain specific. No cross-reactivity of kappa and lambda light chains was demonstrated.

Anti-IgA-secretory-piece.—This was provided by Dr R. A. Thompson (Regional Immunology Laboratory, East Birmingham Hospital). An IgA-secretory-piece antiserum was produced by immunisation in a goat with IgA-secretory piece obtained from human colostrum. The antiserum was then absorbed with human serum IgA to remove any anti-7*S* IgA. On double immunodiffusion no lines of precipitation were obtained when tested against whole human serum, and one line of precipitation obtained when tested against human jejunal juice. For the indirect immunofluorescence studies, a F.I.T.C.-rabbit-anti-goat gammaglobulin conjugate (Nordic) was used at a 1/5 dilution.

Results

IgA was detected in all 34 samples in the region of the dermal papillæ. In the uninvolved specimens, the patterns of immunofluorescence were either "papillary" or "continuous", as previously described.[2] In involved lesions, the pattern of staining was more granular and diffuse (fig. 1). IgA-secretory piece was not detected in any of 4 samples studied. IgM was present in 9 and IgG in 3 of the 34 samples in the same sites.

C3 deposits were present in 16 (47%) of the 34 specimens (table). The patterns of immunofluorescence corresponded to that associated with the IgA deposits (figs. 1 and 2). The highest proportion of C3

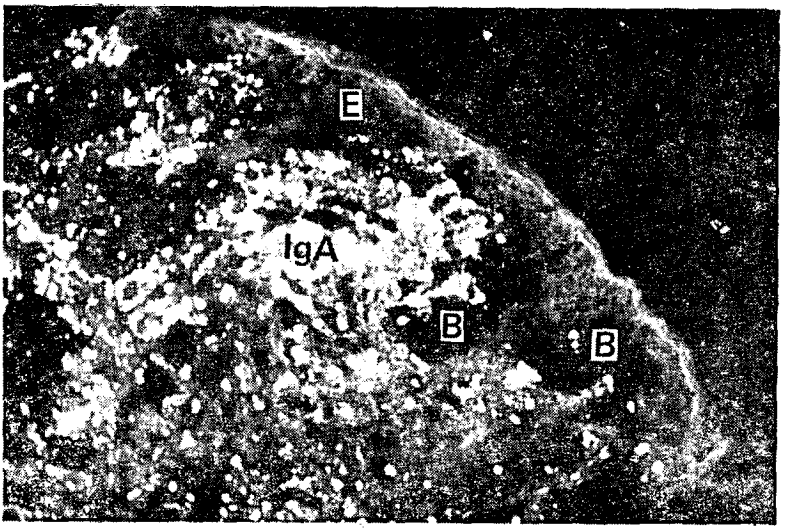

Fig. 1—D.H. lesion showing IgA deposits (IgA) in blister (B) and intact epidermis (E).

Staining with F.I.T.C. anti-C3 showed C3 deposits in same situation as IgA. (Reduced to 2/3 of ×160.)

Fig. 2—Uninvolved D.H. skin showing C3 deposits below the basement membrane in a continuous pattern.

Staining with F.I.T.C. anti-IgA showed IgA deposits in the same situation as C3. (Reduced to 2/3 of ×400.)

deposits (8/9) was in the specimens from the involved (erythematous/peribullous) skin. C3 was also present in the uninvolved skin of 3/9 specimens from patients not receiving any treatment, in 2/8 where the rash was controlled by dapsone, and in 3 of the 8 where the rash was controlled by a gluten-free diet (G.F.D.).

C1q was found in only 3 (9%) of the 34 specimens: in these 3 IgG was also present in 1 and IgM in 2.

Discussion

We found IgA in all 34 skin samples examined and C3 in half of them. Complement may be activated by two main processes—either by the "classical pathway" which includes the first three components of complement (C1, C2, and C4) or by the "alternate pathway" which bypasses these three components and by which C3 is activated as the initial event of the complement cascade.[9] C1q is a subunit of the first component (C1) of human complement, and its presence with C3 in a lesion indicates complement activation by the classical pathway. The absence of C1q deposition in almost all patients suggests that the C3 in the skin in D.H. has been activated by the alternate pathway, and that the activating agent is IgA. In the 3 samples where C1q was detected, the presence of IgA was associated with that of IgG or IgM, both of which activate complement by the classical pathway.

C3 deposition was found in the involved skin lesions in all but one of the nine patients not receiving treatment. C3 was found in 3 of the 9 specimens of uninvolved skin from patients not receiving treatment; 2 of the 8 specimens from patients being treated with dapsone; and in three of the eight patients whose rash was controlled on a G.F.D. These results are difficult to explain, but a number of possibilities exist. First, in the patients not receiving treatment, complement could well have been activated and the biopsy taken before the lesion had fully developed. Dapsone may not block the activation of the complement but instead affect a subsequent process in the formation of D.H. blisters. This is consistent with the observation that membrane-bound vacuoles occur in the uninvolved skin of patients with D.H. taking dapsone.[10] The vacuoles were considered to be an early stage in the blister formation,[11] and pre-

sumably occur after complement activation. Finally in the patients taking a G.F.D., it is possible that the C3 found was in an inactive form, and that activation is dose-related to the quantity or nature of IgA present. Aggregated IgA can activate the alternate pathway,[12] and it requires a higher concentration of complement than does the classical pathway.[13] It may well be that IgA antibody complexed with antigen has the same effect, and in D.H. the formation of such complexes may be related to gluten ingestion. In D.H. circulating immune complexes are present, and the incidence is much higher in patients taking gluten compared to those on a G.F.D.[14] However, the precise nature of these complexes is unknown. In support of the hypothesis that complement is not activated in patients whose rash is controlled by a G.F.D. is the finding that membrane-bound vacuoles are not present in the skin.[10]

Following on the findings of Williams et al.,[15] demonstrating a possible role of the alternate pathway of complement activation in the pathogenesis of mesangiocapillary glomerulonephritis, our findings suggest that this mechanism also occurs in D.H. The significance of IgA deposits in the skin in D.H. has not been previously explained, and our results now indicate a possible way that they may be involved in the disease process. It is possible that the alternate pathway of complement activation also occurs in the small intestine in cœliac disease and plays an essential part in the pathogenesis of the intestinal lesion. IgA and C3 have recently been found in the small intestine of these patients after gluten challenge.[16] However, no studies of C1q or other components concerned in the classical pathway have as yet been carried out on the gut in gluten-sensitive enteropathy. In addition, it has yet to be explained why IgA binds to the reticulin of the dermal papillæ in D.H. but not in cœliac disease. Whether this is due to differences in the skin or in the circulating complexes in the two diseases [14,17] remains uncertain.

We thank Dr R. A. Thompson for providing the IgA-secretory-piece antiserum. P. P. S., L. F., and M. R. M. acknowledge grants from the M.R.C. and Wellcome Trust.

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SALIVARY IgA IN PATIENTS WITH PSORIASIS AND DERMATITIS HERPETIFORMIS

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Abstract. By means of a radial immunodiffusion technique, salivary IgA levels have been estimated in parotid gland saliva in 12 patients with dermatitis herpetiformis, 12 patients with psoriasis and in 12 healthy controls. Significantly elevated values were found in patients with psoriasis (mean 6.85 ± S.E. 1.20 mg/100 ml) compared with the normal controls (mean 3.48 ± S.E. 0.69 mg/100 ml) ($P < 0.05$). In dermatitis herpetiformis the values were not elevated (mean 3.84 ± S.E. 0.50 mg/100 ml).

Abnormalities of serum immunoglobulin have been reported in dermatitis herpetiformis (D.H.) (6, 7). These authors found low serum IgM levels similar to those described in coeliac disease (C.D.) (8). By contrast, serum IgA levels have been reported to be elevated in D.H. (6) and similarly in C.D. by some authors, though others (3) found depressed levels. Despite the elevated serum IgA levels in C.D. and D.H., Beale et al. (1) have recently suggested that there is an impaired IgA response in patients with C.D., possibly related to an abnormality of secretory IgA.

We have, in this study, collected parotid saliva (a good source of IgA) to determine if any quantitative IgA abnormality could be detected in D.H. In addition, because of the reports (8, 16) of elevated serum IgA levels in psoriasis, we have studied salivary IgA in a group of patients with psoriasis.

METHOD

Twelve patients with psoriasis (age range 18–71 years) and 12 with D.H. (age range 25–70) were investigated. Twelve healthy individuals, mainly medical and nursing staff with no history of skin disease or oral infection (aged between 19–50 years) served as controls. Specimens of saliva (stimulated with lemon juice) were collected using Curby cups (4) into plastic containers and stored imediately at −20°C until processed.

Salivary IgA was measured by a modified single radial immunodiffusion technique (10) on commercially available immunoplates (Hyland Low Level Plates from Baxter Laboratories, Thetford, Norfolk). The immunoglobulin was measured by using low strength antisera to IgA in agarose plates. The wells were filled to the brim and allowed to empty completely before being refilled again in the same way. This procedure was performed three times altogether in all samples and suitably diluted standards, thus internally correcting any error resulting from refilling of the wells. The rings of the precipitate were then measured in all samples and standards and plotted on semilog graph paper. From the standard graph it was thus possible to read off the values for the patients' saliva. Salivary albumen levels were measured using a similar immunodiffusion technique.

RESULTS

The levels of salivary IgA in stimulated parotid secretion are shown in Fig. 1.

The mean salivary IgA was 3.48 ± S.E. 0.69 mg/100 ml in the control group and 3.84 ± S.E. 0.50 mg/100 ml in the D.H. group. This is not significantly different. In the patients with psoriasis the mean value was 6.85 ± S.E. 1.20 mg/100 ml. This is a significant increase ($P < 0.05$) compared with the control group.

The albumen levels were in the normal range (< 3 mg/100 ml) in all patients except one with psoriasis in whom it was 12 mg/100 ml. However, the salivary IgA level in this patient was not elevated (3.0 mg/100 ml).

DISCUSSION

These results show that in patients with psoriasis there is a significantly elevated level of IgA in parotid secretion. In patients with D.H. this level was found to be similar to controls. Stimulated

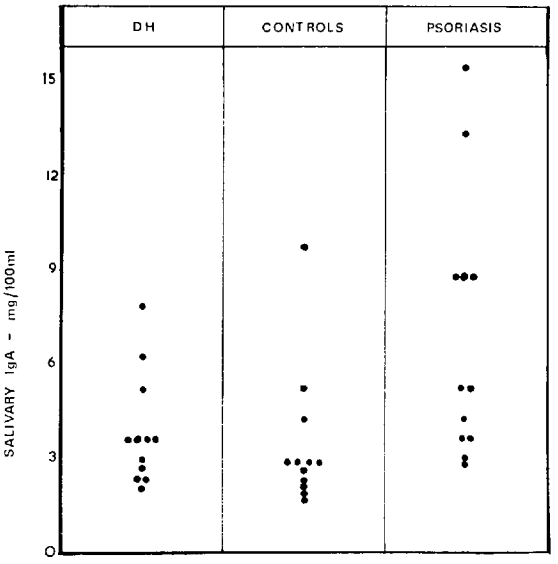

Fig. 1. Salivary IgA levels in dermatitis herpetiformis, psoriasis, and normal controls.

parotid salivary IgA was studied for various reasons. In secretions of the gastrointestinal tract, IgA is the major class of immunoglobulin present. The parotid gland has one of the highest concentrations of IgA plasma cells in the body and also produces higher levels of IgA than any other oral secretion (2). In addition, stimulated saliva provided a better frequency distribution (11). Our mean value of 3.84 mg/100 ml in patients with gluten-sensitive enteropathy (D.H.) is much lower than the 3–16 mg/100 ml range found by Douglas et al. (5) in untreated coeliac disease and < 1–18 mg/100 ml in treated patients. However, as their controls had similarly raised levels this may reflect on their method of collection, as unstimulated specimens are known to have values of IgA as high as 25 mg/100 ml.

Our results in D.H. show normal salivary IgA levels and do not agree with those of McClelland et al. (9) who found elevated levels. Their mean salivary IgA level for 16 patients with D.H. was 10.9 ± 5.5 mg/100 ml compared with 7.0 ± 2.3 mg/100 ml in 16 controls, the difference being significant ($P < 0.05$). These authors also found elevated levels of IgM and IgA in the small intestinal secretions in D.H. and suggested that this finding, together with the possibility of raised IgA levels in saliva, might point to a widespread abnormality in the secretory immunoglobulin system in D.H. patients. Further work is obviously necessary to further elucidate this point.

At present there is no evidence that psoriasis is an immunological disorder, whereas there is now considerable evidence that D.H. is (e.g. IgA deposits on the reticulin fibres in the skin (12, 15), and serum anti-reticulin and anti-nuclear antibodies (13)). Although elevated serum IgA levels have been reported in psoriasis (6, 16), no explanation for this finding has been put forward. The elevated salivary IgA reported here may be due to simple diffusion of 7 S IgA globulin (the predominant form in the serum.) However, Fraser et al. (6) found that the serum levels of IgA were raised to similar levels in both D.H. and psoriasis. It would therefore seem unlikely that the raised parotid IgA in psoriasis is due to simple diffusion. At the present time the site of production of the elevated serum IgA levels in D.H. and psoriasis is unknown and the modified radial immunodiffusion technique does not differentiate between 7 S and secretory 11 S IgA. Thus, we are unable to ascertain whether the elevated IgA in the parotid secretion is 7 S and due to simple diffusion, or 11 S as a result of increased production by the salivary gland. However, to exclude possible leakage of 7 S IgA into saliva due to unsuspected inflammation, we have measured the salivary albumen which is known to be increased in inflammation (14). This was only above normal in one patient with psoriasis and thus cannot explain our findings in the group of patients with psoriasis. It is obvious that techniques to distinguish between 7 S and 11 S globulin should be employed to define the type of IgA and thus demonstrate where the abnormality lies in psoriasis, as compared with normal persons.

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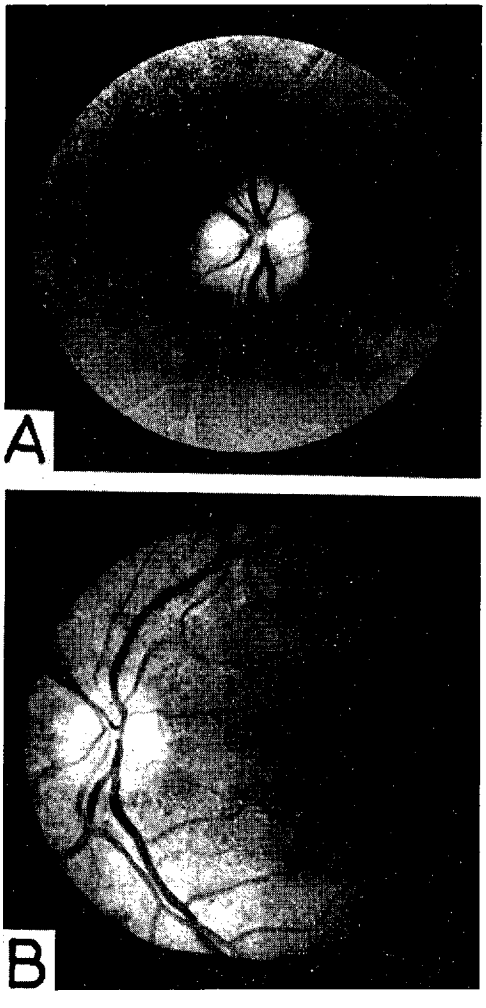

Fig 1 *Fluorescein fundus photographs, right eye (residual films).* A, *November 1970.* B, *March 1971*

Walsh & Hoyt (1969) quote, but without details, the case of William Spencer in which optic neuritis and carcinoma of the bronchus were associated. There was no case of optic neuritis in the London Hospital series of patients with neurological complications of the reticuloses (Currie & Henson 1971).

The alternative hypothesis is that the patient has two unrelated conditions, namely a uveomeningitic syndrome and carcinoma of the breast. Thus sarcoidosis could cause the CSF pleocytosis, but the retinitis is unlike that seen in this condition, macular involvement and papillitis being rare (Geeraets *et al.* 1962), and there was no other evidence of sarcoid on chest X-ray or lymph node biopsy. The Vogt-Koyanagi-Harada syndrome can produce a picture very similar to that in this patient but it would be unusual for the skin, hair and eighth nerve function all to be completely normal after two years (Cowper 1951). Similarly, it is difficult to sustain a diagnosis of Behçet's disease in the absence of mucocutaneous manifestations after such an interval. It would thus seem unlikely that this patient suffers from any of these syndromes. That her neurological condition is related to the neoplasm is the most plausible hypothesis.

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Gluten-sensitive Dermatitis Herpetiformis

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and J S Stewart MD MRCP (*West Middlesex Hospital, Isleworth, Middlesex*)

Mrs P P, aged 39

History: She was admitted to the West Middlesex Hospital in March 1960 aged 26 (Dr N F Coghill) with 8 years recurrent colicky abdominal pain and one or two loose, pale, offensive stools per day. Three episodes of unexplained anæmia. Fair appetite, steady weight, no vomiting. No history of childhood cœliac disease but one year previously had been thought on clinical grounds elsewhere to have cœliac disease for which she had been tried on a brief partial gluten-free diet without benefit. No drugs, chest or skin trouble or abdominal operations and never in tropics. No physical signs except that she appeared anæmic.

Investigations: March 1960: Hb 8.2 g/100 ml; hypochromasia. Sternal marrow moderately megaloblastic, no iron stores; still megaloblastic following trial of vitamin B_{12} to confirm folate deficiency (folate assays not then available). Folic acid absorption 4:18:47 ng/ml after oral dose following 'saturation' (normal peak rise >40 ng/ml). Plasma calcium 9.2 mg/100 ml, alkaline phosphatase 23 K-A units/100 ml, albumin 4.0 g/100 ml, globulin 2.7 g/100 ml. Xylose excretion 2.8 g in 5 hours following 25 g oral dose (normal >5 g). Glucose tolerance test 64:68:88:

75:80 mg/100 ml at half-hourly intervals (normal peak rise >40 mg/100 ml). Daily fæcal fat 9g (6 day collection). Barium follow through normal. Jejunal biopsy: flat mucosa, cœliac lesion histologically, surface cell height 15 μm (normal >29 μm). Cœliac disease was diagnosed and treated at first with oral iron and folic acid (patient reluctant to take diet).

October 1960: started gluten-free diet and felt better (Hb 11.6 g/100 ml), but returned to normal diet in February 1961; remained fairly well until Hb fell to 9.2 g/100 ml and serum folate to 3.0 ng/ml in 1963; restarted on oral iron and folic acid. Remained on this treatment intermittently throughout 1960s.

1967: seen during a survey by a dermatologist who found nothing abnormal. 1968: first noticed clear itchy blisters on elbows, front of knees and thighs. 1970: iron absorption (whole body counter 14 days retention) 0.8% (normal >8.0%) and mean daily free iron loss 0.163% over 21 days (normal 0.018–0.026%) (Dr D Sutton).

March 1971: sore mouth and tongue. Hb 12.8 g/100 ml; macrocytosis. ESR 6 mm in 1 h (Wintrobe), WBC 3000/mm^3 (neutros. 40%, lymphos. 54%, eosinos. 4%). Serum B_{12} 80 pg/ml, folate 3.2 ng/ml, calcium 8.2 mg/100 ml, phosphate 2.8 mg/100 ml, alkaline phosphatase 107 iu/l. (normal 20–110 iu/l.), serum immunoglobulins normal (IgG 1140, IgA 130, IgM 138 mg/100 ml). Repeat jejunal biopsy: flat as before. Started strict gluten-free diet: morphological improvement (*see* Chapman *et al.* 1973).

September 1971: included in immunofluorescent study of skin of patients with dermatitis herpetiformis (DH) (Dr L Fry). IgA deposits found in dermal papillæ (Fig 1).

1971–73: good clinical and objective response of cœliac disease to strict diet. DH also controlled by gluten-free diet alone, recurring briefly on two occasions when she lapsed from the diet. She never had dapsone.

Discussion

The association of a gluten-sensitive small bowel enteropathy with DH is now well established (Marks *et al.* 1966, Fry *et al.* 1967, Brow *et al.* 1971). This patient has both features, but is unusual in that symptoms from her enteropathy long preceded those of her skin disorder. This is in contrast to the majority of patients with DH who usually present to dermatologists first, though there have been reports of the enteropathy preceding the skin eruption (Fraser *et al.* 1968, Shuster *et al.* 1968). A further unusual feature is that symptoms from the skin eruption have been minimal, and she was only picked up as a case of DH when she was included in an immunofluorescent study of the uninvolved skin in patients with

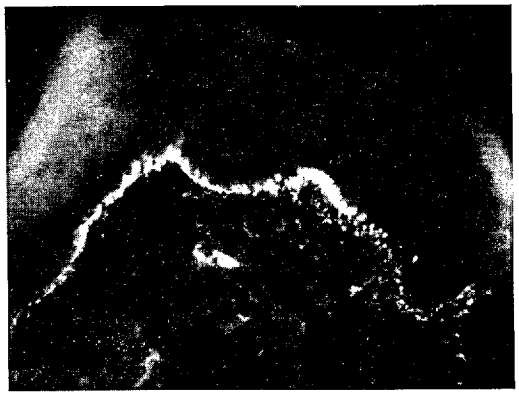

Fig 1 *IgA deposits in dermal papillæ of uninvolved skin detected by direct immunofluorescence.* ×*180*

DH and adult cœliac disease (Seah *et al.* 1972); she was the only one of 22 patients with adult cœliac disease to have IgA deposits in the dermal papillæ in her uninvolved skin. Subsequent studies (Fry & Seah 1973) show that this finding is diagnostic for DH. The patient, in retrospect, gives a history of a skin rash characteristic of DH. She has, however, never had to take dapsone or sulphapyridine to control her rash which is now completely quiescent on her strict gluten-free diet. Fry *et al.* (1973) have recently reported the efficacy of such a diet in controlling the skin rash of DH, and suggest that this therapy should be considered in all patients with this condition.

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Sir Robert Drew said that they were all interested to learn of the characteristic apperances in immunofluorescent studies of the skin in this disease. One explanation of these deposits could be that IgA was absorbed through the gut and deposited in the dermal papillæ. Would Dr Seah agree that in the past a number of cases had been wrongly labelled as dermatitis herpetiformis and treated with dapsone?

Dr Seah agreed. A recent study (Fry & Seah 1973) found that in 13 of 58 patients a clinical diagnosis of DH could not be substantiated on further testing.

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Pemphigus controlled by sulphapyridine

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SUMMARY

A patient with an 11-year history of a generalized blistering eruption is described. The eruption was completely controlled within 1 week of taking sulphapyridine and recurred within 4 days of withdrawal of this drug. On account of these observations a diagnosis of dermatitis herpetiformis was made. Histological, immunological (and small intestinal) studies now show that this patient had pemphigus.

Dapsone and sulphapyridine both control the eruption of dermatitis herpetiformis. The response is so dramatic that a therapeutic trial of either drug is often used in the diagnosis of this condition. There have been reports of four patients (Floden & Gentele, 1955; Doepfner, 1960; Winklemann & Roth, 1960) in whom the diagnosis of dermatitis herpetiformis had been queried, as these patients had a blistering eruption which was controlled by sulphonamides though the histology of the lesions showed the blisters to be intraepidermal. We report a patient whose blistering eruption was controlled by sulphapyridine and, as a result, was diagnosed as having dermatitis herpetiformis (even though histologically the blister was intraepidermal), but now immunological studies have shown the patient to have pemphigus and no evidence of dermatitis herpetiformis.

CASE REPORT

History

The patient was a female Caucasoid aged 43. 11 years ago, at the age of 32, she developed an irritating eruption, which she described as 'spots', on her shoulders and abdomen. There were no blisters at this time. Her general practitioner initially diagnosed ringworm infection and, subsequently, pityriasis versicolor. Her eruption failed to respond to a number of ointments and she developed blisters a few weeks later on the trunk and shoulders; she was then referred to St Bartholomew's Hospital, Rochester. A clinical diagnosis of dermatitis herpetiformis was made and a biopsy carried out. The biopsy was reported by the Histopathology Department at St John's Hospital for Diseases of the Skin as follows: 'There are two intra-epidermal bullae filled with eosinophils and one bulla shows acantholysis, which does not imply that the case is pemphigus vulgaris'. The patient was initially treated as for dermatitis herpetiformis with dapsone, 200 mg/day, and the eruption cleared within 2 weeks. However, 3 months later, whilst she was still taking this dose of dapsone, the rash returned. When the dose of dapsone was increased to 300 mg/day she felt unwell and became 'blue'. This was shown to be due to methaemoglobinaemia. The dapsone was stopped and she was given a 6 week

course of systemic steroids with little improvement. Following this she was given sulphapyridine 1·5 g/day and this controlled the eruption. She has now been taking this drug for 10 years, although the dose needed for control of the eruption is now 3·0 g/day.

In 1972 she was referred to the dermatitis herpetiformis clinic at St Mary's Hospital for reassessment and possible treatment by a gluten-free diet. Physical examination showed no abnormality apart from numerous small scars. Sulphapyridine therapy was stopped, and 3 days later the patient developed a vesicular eruption on the limbs and trunk. The rash subsided within a week of recommencing the sulphapyridine, and on stopping this drug a second time she again developed blisters within 4 days.

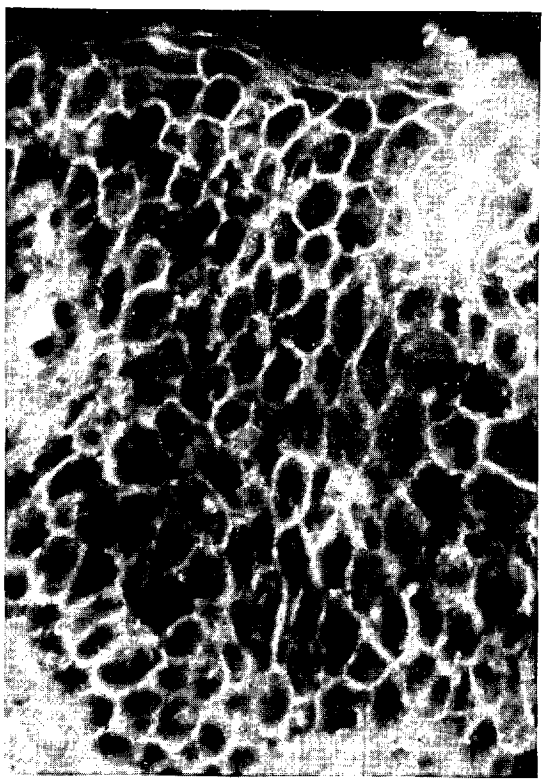

FIGURE 1. High power view of positive epidermal intercellular fluorescence. Section of skin taken adjacent to bullous lesion stained with FITC–sheep–anti–human IgG for direct immunofluorescence (× 800).

Investigations

Haematological. Hb 13·1 g/100 ml, WBC 7100/mm^3, serum folate 8 ng/ml, red cell folate 506 ng/ml, serum vitamin B_{12} 564 pg/ml, serum iron 195 μg/100 ml, bone marrow biopsy normal apart from no stainable iron.

Gastro-intestinal. Faecal fat excretion 0·7 g/day, small intestinal biopsy: macroscopic, fingers and leaves; microscopic, normal villi with columnar epithelium; epithelial lymphocyte count, 253/thousand epithelial cells.

Skin biopsy. This showed an intradermal blister containing acantholytic cells and eosinophils. The level of the blister is predominantly subcorneal.

Immunological (direct immunofluorescent studies). These studies were carried out as described by Seah *et al.* (1972).

Class specific fluorescein isothiocyanate (FITC) conjugated sheep-anti-human IgG, IgM and IgA (Wellcome Reagents) and FITC-goat-anti-human $\beta 1_c/\beta 1_a$ (complement C_3) (Hylands) were used. Controls were provided by incubating the patient's skin sections with FITC conjugated sheep-anti-rabbit anti-serum (Wellcome Reagents) and by incubating sections of skin from a normal person with the same conjugates. Specimens were examined on a Reichert 'Diapan' microscope with a quartz iodine light source, a Balzer FITC 3 primary interference filter and a Wratten 12 secondary filter.

Strong staining of the intercellular spaces in the epidermis was seen with anti-IgG (Fig. 1). No IgM, IgA or $\beta 1_c/\beta 1_a$ deposits were detected in the dermis or epidermis. There was no evidence of basement membrane staining. No immunofluorescent staining was seen with the controls.

Indirect immunofluorescent studies. A polyvalent FITC sheep-anti-human immunoglobulin conjugate (Wellcome Reagents) was used for these tests.

Intercellular antibody (Beutner & Jordon, 1964) was detected at a titre of 1 : 10. There was a positive anti-nuclear factor at a titre of 1 : 80. No basement membrane (Beutner *et al.*, 1965), reticulin, smooth muscle, mitochondrial or gastric parietal cell antibodies (Seah *et al.*, 1971) were detected.

DISCUSSION

This report shows that the patient has pemphigus and not dermatitis herpetiformis, and the disorder is controlled by sulphonamides. The report made 10 years ago by the histopathologist, that the findings do not imply pemphigus, cannot be accepted. The presence of eosinophils in the involved epidermis in patients with pemphigus has been stressed by Emmerson & Wilson-Jones (1968) and cannot, as an isolated finding, be considered to favour a diagnosis of dermatitis herpetiformis.

Certainly in the past, and in the majority of dermatology departments even now, the response of a blistering eruption to sulphonamides and/or dapsone has been taken to imply a diagnosis of dermatitis herpetiformis. However, this assumption must now be seriously questioned, as although the rash of dermatitis herpetiformis does respond to dapsone and sulphonamides, there are reports of other dermatoses, particularly vasculitis and erythema elevatum diutinum (Johnston, 1964; Kalkoff, 1968; Vollum, 1968) responding to these drugs. There have been reports of four patients similar to ours with an eruption of histologically intra-epidermal blisters, controlled by sulphonamides (Floden & Gentele, 1955; Doepfner, 1960; Winklemann & Roth, 1960). All these three reports queried whether these four patients had dermatitis herpetiformis or pemphigus.

Over the last few years definite immunological criteria for diagnosing pemphigus and dermatitis herpetiformis have evolved. The previous reports of this rare group of patients with intra-epidermal blisters showing acantholysis on microscopy, yet responding to sulphonamides have not included immunological findings (although Dobmeier, Mitchell Sams & Beutner (1971) have reported a patient with circulating antibodies characteristic of pemphigus whose eruption responded to sulphonamides, but no intra-epidermal blisters were ever found on microscopy). Our patient showed both *in vivo* intercellular antibodies in the epidermis (as described by Beutner *et al.*, 1965) and circulating antibodies to intercellular cement substance of epidermal cells (as described by Beutner & Jordon, 1964), which may be considered necessary criteria for the diagnosis of pemphigus. Moreover, there

were no IgA deposits in the dermal papillae of the uninvolved skin, such as are present in patients with dermatitis herpetiformis (Van der Meer, 1969; Chorzelski *et al.*, 1971; Seah *et al.*, 1972). Further evidence that this patient does not have dermatitis herpetiformis comes from the investigation of her gastrointestinal tract and folate status. 95% of patients with dermatitis herpetiformis show abnormality of the small intestinal epithelium, which is due to gluten sensitivity (Fry *et al.*, 1972). The most sensitive test for gluten sensitivity is the degree of lymphocytic infiltration of the small intestinal epithelium (Fry *et al.*, 1972). In our patient the macroscopic appearance of her small intestine was normal and her lymphocyte count was 253. This is a high normal figure well below the range of 451 ± 70 found in patients with dermatitis herpetiformis. In addition, 90% of patients with dermatitis herpetiformis have a low serum folate (Fry *et al.*, 1972), and our patient had a normal serum level.

Thus, this is a further report of the control by sulphonamides of the skin eruption of a patient with pemphigus, and the first report of such a patient in whom the diagnosis of pemphigus has been established by immunological studies. The mechanism of action of the sulphonamides in this disorder remains unknown, but we presume it is a block of an immunological reaction. Unfortunately, at the present time, there are no further investigations which can distinguish this rare group of patients with pemphigus, whose rash is controlled by sulphonamides, from the majority who require other more potent and dangerous drugs.

ACKNOWLEDGMENTS

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Dermatitis herpetiformis: an evaluation of diagnostic criteria

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SUMMARY

Forty-two patients in whom a clinical diagnosis of dermatitis herpetiformis (DH) had been made, were studied. All patients had a small intestinal biopsy, a biopsy of uninvolved skin for detection of the presence of IgA deposits by immunofluorescence, serum and red cell folate estimations and examination of the serum for anti-reticulin antibody. The response of the skin eruption to dapsone was noted. Thirty patients had biopsies of skin lesions for routine histological examination.

IgA deposits were found in the uninvolved skin of thirty-five of the forty-two patients. Evidence of an enteropathy was found in thirty-four of those thirty-five patients. Of the seven patients who did not have IgA in the uninvolved skin, only one showed evidence of enteropathy, which was slight, and this patient was shown subsequently to have pemphigus and not DH. Of the thirty biopsies of skin lesions, only five showed all the features usually considered to be characteristic of DH. Anti-reticulin antibody was found in eight patients, and in all of these IgA was present in the uninvolved skin. Low red cell folate levels were found in eleven patients (all of whom had an enteropathy and IgA in the uninvolved skin). Low serum folate levels were found in thirty-four patients, thirty-one of whom had IgA deposits in the uninvolved skin and an enteropathy; in the other three patients who did not have IgA deposits or an enteropathy, all were on dapsone and this may have accounted for the abnormal result.

In forty-one of the forty-two patients the rash was dapsone responsive. The patient who showed no response to dapsone had no IgA in the uninvolved skin and no evidence of enteropathy. Follow-up showed that of the seven patients with no IgA in the skin, one had pemphigus, and in three the rash eventually cleared spontaneously and the patients no longer required treatment. There was no evidence of spontaneous remissions in the patients with IgA in the uninvolved skin.

It would appear that the presence of IgA deposits in the uninvolved skin is part of the syndrome of DH and that the diagnosis should not be made unless these deposits are found.

Dermatitis herpetiformis (DH) has on clinical grounds been known as one of the so-called bullous disorders. The criteria for making the diagnosis of DH and distinguishing it from the other bullous disorders have been arbitrary and empirical. In his review of the diagnosis of DH, Smith (1966) stated that there is no 'universal agreement as to the criteria for making the diagnosis of DH'. Until 1967 it mattered little as to the exactness in diagnosis as the treatment was empirical, and nothing was

known of the cause of the disorder. In fact, the commonest way of establishing the diagnosis was, and still is in the majority of dermatological departments, empirically to assess the response of the rash to dapsone or sulphonamides. This is unsatisfactory, as it is now known that some other skin disorders also clear with dapsone.

However, following preliminary reports of an enteropathy in DH (Marks, Shuster & Watson, 1966; Fraser, Murray & Alexander, 1967; Fry *et al.*, 1967), it has been shown that this enteropathy is due to gluten sensitivity (Fry *et al.*, 1967, 1969; Shuster, Watson & Marks, 1968), that all patients with DH have this enteropathy (Fry *et al.*, 1972), and that the skin rash is also gluten dependent (Fry *et al.*, 1968, 1969, 1973). Thus, there is now a specific treatment for patients with DH, namely a gluten-free diet (GFD). Although this is a harmless treatment compared to many others given by the medical profession today, it does impose a very real social handicap on the patients, and it is without question very difficult for a large proportion of the patients to adhere strictly to a GFD. If a GFD is to be successful in the clearing of skin lesions in DH, it must be strict (Fry *et al.*, 1973) and, at the present time, must be adopted for life. Thus, before embarking on this treatment it is most important to establish the correct diagnosis. In our opinion the usual criteria of clinical and histological features, and response to dapsone, are not precise enough in establishing the correct diagnosis in all patients.

A number of immunological abnormalities in patients with DH have recently been described. These include the presence of circulating anti-reticulin (ARA) and anti-nuclear antibodies (Seah *et al.*, 1971a), IgA deposits in the skin (Van der Meer, 1969; Chorzelski *et al.*, 1971; Seah *et al.*, 1972), and circulating immune complexes (Mowbray *et al.*, 1973). A high incidence of folate deficiency (Fry *et al.*, 1967; Brow *et al.*, 1971) and splenic atrophy (Pettit *et al.*, 1972) have also been described. Thus, with a clearer understanding of the cause of DH and a number of the associated pathological abnormalities, we have attempted to assess the most useful criteria for establishing the diagnosis.

PATIENTS AND METHODS

Patients

Forty-two patients (aged 22–75 years), comprising twenty males and twenty-two females, were studied. They were referred with a clinical diagnosis of 'dermatitis herpetiformis' from nine consultant dermatologists over a period of 6 years.

Methods

The patients were assessed by the following seven criteria.

(*a*) *Clinical features.* The clinical features that were present at the first consultation with the referring consultant were noted. The features usually considered to be characteristic of DH are intensely irritating grouped blisters on the knees, elbows, buttocks, scapulae and other areas which may be subjected to pressure. The blisters frequently arise on an urticarial base. Because of the intense irritation only grouped excoriated papules are sometimes seen at the above sites. The rash may therefore be considered to be polymorphic, as originally described by Duhring.

(*b*) *Response of rash to dapsone and sulphapyridine.* The response of the skin rash to dapsone or sulphapyridine was assessed. Initially patients were given 100 mg of dapsone per day; if there was no improvement the dose was increased to 200 or even 300 mg/day. Patients were seen at weekly intervals. Dapsone was the drug of first choice and sulphapyridine was used only when patients could not tolerate dapsone. The dose of sulphapyridine varied from 1 to 3 g/day. In patients whose rash cleared, therapy was withdrawn to see if relapse of the rash occurred.

(*c*) *Histology of skin lesions.* Skin biopsies had been carried out on thirty of the forty-two patients. Vesicles or early erythematous lesions were taken under local anaesthesia and processed for routine histology. An appraisal as to why so few patients had had biopsies indicated that, whilst absence of suitable lesions for biopsy was a factor, this was minimal, and that in the main most of the dermatologists elected to accept the clinical features and response to dapsone and/or sulphapyridine as sufficient evidence for the diagnosis of DH, and not to perform a biopsy.

The histological features were assessed according to the criteria of Pierard & Whimster (1961) and graded according to the histological features considered to be characteristic of DH. These included the presence of sub-epidermal bullae, papillary micro-abscesses and infiltration with neutrophils and eosinophils. For the purpose of this study, biopsies were assessed according to the presence of these features. A biopsy showing all three features was scored + + +, with two + +, with one +, and o for none.

(*d*) *Anti-reticulin antibody.* An autoantibody directed against reticulin has been found in patients with DH (Seah *et al.*, 1971a) and in patients with coeliac disease (Seah *et al.*, 1971a, 1971b; Alp & Wright, 1971; Von Essen, Savilahti & Pelkonen, 1972). The presence of this antibody was looked for by indirect immunofluorescence according to the method of Seah *et al.* (1971a). A sheep-anti-human immunoglobulin fluorescein (FITC) conjugated anti-serum (Wellcome Reagents, lot K.4761, F/P ratio: $OD_{495:280}$–0·85, molar 3·7) was used at a working dilution of 1:32.

(*e*) *IgA in skin.* This was detected by direct immunofluorescence as described by Seah *et al.* (1972). Clinically uninvolved skin was taken from the buttocks using a 3 or 4 mm punch biopsy under 2% lignocaine local anaesthesia, and embedded in OCT (Lab. Tek, Indiana) in pre-cooled isopentane. 6 μm sections of skin were cut in a cryostat, transferred onto glass slides and air-dried. The slides were prewashed in phosphate buffered saline (PBS) pH 7·2 and then stained with the anti-IgA fluorescein conjugate for 30 min. After a final wash for 1 h in PBS pH 7·2, they were mounted with buffered glycerol, pH 8·0, and examined on a Riechert 'Diapan' microscope with a quartz–iodine light source, a Balzer FITC-3 primary interference filter and a Kodak Wratten 12 secondary filter. Biopsies were taken irrespective of whether the patient was on therapy (dapsone, sulphapyridine or a gluten-free diet) or not, and in those patients with skin lesions, care was taken to obtain the biopsies from clinically uninvolved areas well away from the lesions. A commercial sheep-anti-human IgA fluorescein (FITC) conjugate (Wellcome Reagents, lot K.5777, F/P ratio: $OD_{495:280}$–0·71, molar 3·3) was used at a dilution of 1:12. Prior testing of the conjugate against α-chain monoclonal bone marrow was positive, and negative results were obtained with γ and μ heavy chain and k and λ light chain monoclonal bone marrow.

Controls were performed using uninvolved skin from normal volunteers, and by staining some of the DH biopsies with sheep-anti-rabbit immunoglobulin fluorescein (FITC) labelled antisera (Wellcome Reagents, Lot K.5863). Blocking experiments were performed using an unconjugated sheep-anti-human IgA antiserum (Wellcome Reagents).

(*f*) *Small intestinal biopsy.* This was performed using a Crosby capsule under radiographic control. Biopsies were taken at the level of the ligament of Trietz. Specimens were examined on a dissecting microscope and then fixed in formol saline and embedded in wax. 7 μm sections were cut and stained with haematoxylin and eosin.

Macroscopic appearance. Under the dissecting microscope, the appearance of the biopsies was classified into four main categories: flat, convoluted, leaves only, and fingers and leaves. Flat and convoluted specimens were considered to be abnormal.

Microscopic appearance and lymphocyte count. At the microscopic level, sections of the jejunal biopsies were examined for evidence of infiltration with lymphocytes. The number of intra-epithelial lymphocytes was quantified in each specimen, as described by Fry *et al.* (1972), as an index of this lymphocytic infiltration, and expressed as intra-epithelial lymphocytes per 1000 epithelial cells. A count above 200 is suggestive of an abnormality. A count above 300 is definitely abnormal.

(*g*) *Folate status.* Serum folate levels were estimated using *Lactobacillus casei* as the test organism (Waters & Mollin, 1961). The normal range is 6·0–21·0 ng/ml. Levels less than 3·0 ng/ml were regarded as abnormal, and those between 3·0 and 5·9 ng/ml as borderline. Red blood cell folate levels were estimated according to the modification of the serum folate assay described by Hoffbrand, Newcombe & Mollin (1966). The normal range is 160–640 ng/ml packed red blood cells.

RESULTS

(*a*) *Clinical features*

Only fifteen (36%) of the forty-two patients had on their first consultation at a dermatological clinic the typical features of dermatitis herpetiformis which enabled the diagnosis to be made with certainty on this occasion.

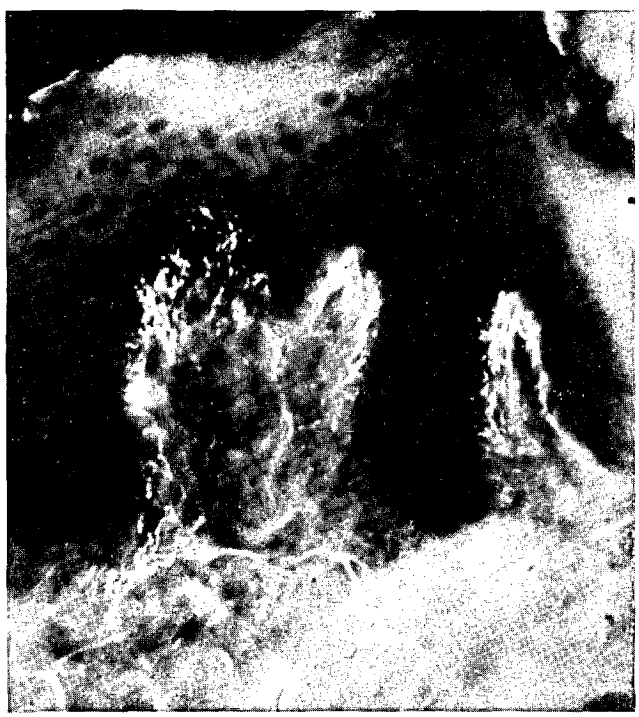

FIGURE 1. Uninvolved DH skin—direct immunofluorescence showing 'papillary' IgA deposits in the dermal papillae (× 400). (By permission of the editor of the *Proceedings of the Royal Society of Medicine.*)

(*b*) *Response of rash to dapsone or sulphapyridine*

The skin lesions improved with dapsone in forty-one of the forty-two patients and the eruption recurred when the drug was withdrawn. Three patients whose rash responded favourably to dapsone were unable to tolerate this drug, and in two of these the eruption was controlled by sulphapyridine.

(*c*) *Histology of skin lesions*
Of the thirty biopsies, five (17%) had all three histological features (+ + +) characteristic of DH, seven (23%) had two (+ +), 16 (53%) had one (+), and two (6%) no features (0).

(*d*) *Anti-reticulin antibody*
This antibody was detected in eight (19%) of the forty-two patients. Seven of these patients were on a normal diet and one on a gluten-free diet.

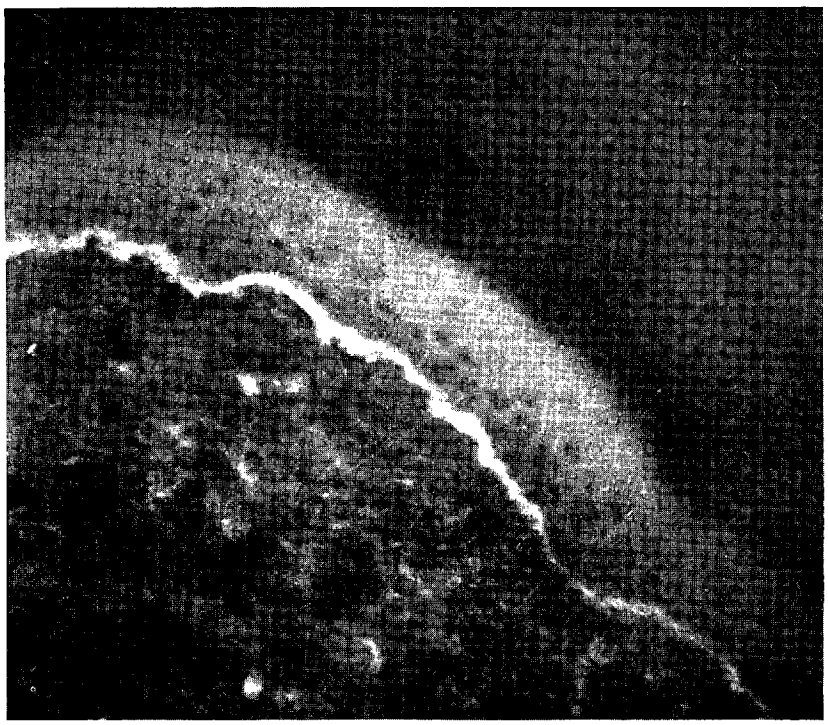

FIGURE 2. Uninvolved DH skin—direct immunofluorescence showing 'continuous' pattern of IgA staining (× 160).

(*e*) *IgA in skin*
IgA deposits were detected in thirty (83%) of the forty-two patients, in the dermis of the uninvolved skin. These deposits were most commonly seen (in thirty-one of the thirty-five) as fibrillar fibres in the tips of the dermal papillae (Fig. 1), and less frequently (in four of the thirty-five patients) as a continuous line (Fig. 2) at the dermo-epidermal junction. No positive immunofluorescence was seen with skin from normals, or with the control fluorescein conjugated anti-serum when tested against the DH skin biopsies. The specificity of the positive IgA staining was substantiated by the ability of unconjugated anti-sera to abolish the positive staining with FITC-conjugated anti-human IgA.

(*f*) *Small intestinal biopsy*
Macroscopic appearance. In the forty-two patients the macroscopic appearance of the small intestine was abnormal in seventeen. It was flat in nine and convoluted in eight. In the remaining twenty-five biopsies, the appearances were leaves only in six, and fingers and leaves in nineteen.

Intestinal epithelial lymphocyte count. The lymphocyte count was below 200 in eight of the forty-two biopsies. Of the nine flat biopsies, all had counts above 200. Of the eight convoluted biopsies, one had a count below 200, being 130. This particular patient had a lymphoma of the small intestine. Of the six biopsies which showed leaves only, one had a count below 200. Of the biopsies showing fingers and leaves, six of the nineteen biopsies had counts below 200.

(*g*) *Folate status*

Serum folate. Thirty-four of the forty-two patients had low serum folate levels. In twenty the level was below 3 ng/ml.

Red blood cell folate. Eleven of the forty-two patients had low red blood cell folate levels.

CORRELATION AND ANALYSIS OF RESULTS

The results show that the incidence of a typical clinical presentation of DH in this series was very low. A study of the notes shows that the diagnosis of DH was considered in the majority of patients only after a number of attendances at the skin clinic. Likewise, a routine biopsy showed all the features characteristic of DH in less than 20% of patients.

The criterion which eventually suggested the diagnosis in forty-one of the forty-two patients was the response of the eruption to dapsone. In the one patient who failed to improve with dapsone, the clinical features were considered compatible with and suggestive of DH. This was a woman of 40 with a 6-year history of grouped, irritating papules on the legs and trunk.

The two objective investigations showing the highest incidence of abnormality were the presence of IgA in the uninvolved skin (thirty-five of the forty-two patients) and evidence of gluten-sensitive enteropathy (thirty-five of the forty-two patients). There was good correlation between these two findings. Of the thirty-five patients with IgA in the skin, thirty-four had evidence of a gluten-sensitive enteropathy demonstrated by an abnormal macroscopic appearance of the mucosa and/or abnormal lymphocytic infiltration of the small intestinal epithelium. The one patient who did not have an abnormal small intestinal mucosa macroscopically or increased lymphocytic infiltration (a count of 89) but had IgA in the uninvolved skin, was a man of 55 with a 10-year history of a widespread irritating vesiculo-papular eruption which responded to dapsone. In view of our finding of one other patient with a normal lymphocyte count in the small intestinal epithelium (130) but a convoluted pattern on biopsy and who had a lymphoma of the small intestine, it is possible that our patient with a count of 89 has a basic abnormality of his lymphocytes, and may also be a candidate for a lymphoma.

Of the seven patients who did not have IgA in the skin, only one had an abnormality of the small intestine, which was slight, manifested by a lymphocyte count of 253. This patient was a woman of 43 with an 11-year history of a blistering eruption controlled by sulphapyridine. On further investigation she was found to have pemphigus substantiated by histological (intra-epidermal blister with acantholysis) and immunological studies (circulating and *in vivo* antibodies bound to the intercellular cement substance of the epidermis).

Thus all but one of our patients with IgA in the skin had definite evidence of an enteropathy, and only one of our patients who did not have IgA in the skin (and who was subsequently shown to have pemphigus) showed an abnormality of the small intestine which was slight. Thus there is nearly 100% correlation between IgA in the skin and evidence of an enteropathy, and these appear to be the best diagnostic criteria for DH at the present time.

In six of the seven patients with no IgA in the uninvolved skin, the rash responded favourably to dapsone in a manner similar to those with IgA in the uninvolved skin. Of those six patients, one has

been shown to have pemphigus, but no diagnosis has been made in the other five. However, in three of these five patients, the rash has cleared after intervals of 6 months, 2 years and 5 years respectively. These patients no longer require dapsone. In the past these patients would undoubtedly have been labelled spontaneous remissions of DH, but in our opinion these patients have never had DH. None of our patients with IgA in the skin has ever shown evidence of spontaneous remission, and the duration of their skin disorder varies from 2 to 50 years.

Of the thirty-five patients with IgA in the skin, twenty-nine had low serum folate levels and eleven low red cell folate levels. Of the seven patients who did not have IgA in the skin, none had low red cell folate levels, but three had low serum levels. All these three patients were on dapsone at the time of study and this may have been responsible for this finding. A low red cell folate in association with an enteropathy and IgA in the skin is supportive evidence of DH, but in general its incidence is too low to be recommended as a useful screening test.

DISCUSSION

The results show that at the present time the most reliable criterion for the diagnosis of DH is the presence of IgA in the uninvolved skin. The finding is compatible with the concept that DH is a specific immunological disorder, although the exact significance of these IgA deposits is at present unknown.

In this particular series, IgA was found in thirty-five of our forty-two patients. Of these thirty-five patients, further substantiation of the diagnosis of DH was provided by the finding of a small intestinal abnormality in thirty-four. It is now well established that patients with DH have a gluten-sensitive enteropathy (Fry *et al.* 1972). In the one patient showing no evidence of enteropathy, the possibility exists of a further abnormality of the immune system; that associated with a tendency to develop malignancy. Of the seven patients who did not have IgA in the skin, only one showed a slight abnormality of the small intestine and this patient was subsequently shown conclusively to have pemphigus and not DH. There appears, therefore, to be a good correlation between IgA in the skin and the presence of a gluten-sensitive enteropathy.

In the previous reports of IgA in the uninvolved skin in DH, Van der Meer (1969) found it in only ten of twelve patients studied, but Chorzelski *et al.* (1971) reported its presence in all nineteen patients with 'typical' DH, and in none of their eight patients with 'atypical' DH. However, these latter authors did not specify their criteria for judging a DH patient as being 'typical' or 'atypical'. In the twelve patients with DH reported by Van der Meer (1969), only five were considered to have typical DH by the author and in all five IgA was found in the skin. The remaining seven patients were classified as 'atypical' DH. IgA deposits were found in five of these seven patients. It is possible that the two patients in whom IgA was not found did not have DH. Unfortunately, no other collaborative evidence, such as small intestinal findings and folate status, were discussed to clarify this point.

The presence of ARA has been reported in the sera of patients with gluten-sensitive enteropathy—both in DH and in childhood and adult coeliac disease (Seah *et al.* 1971a, 1971b). In the present study ARA was found in the sera of eight of the forty-two patients. All these eight patients had IgA in the skin. The presence of ARA is further evidence for the diagnosis of DH, but as the incidence of the antibody in the serum is relatively low, its absence does not exclude the diagnosis. The low incidence of ARA in this series may be due to the fact that many of the patients were on a gluten-free diet; it is known that the antibody disappears from the serum following treatment with a GFD and that the incidence is always higher in patients with DH or coeliac disease when they are on a normal diet (Seah *et al.* 1973a).

It is evident from this study that not many patients with DH present with a characteristic clinical pattern. In this series, only fifteen of the forty-five patients had a diagnostic clinical presentation. All

these fifteen did in fact have IgA in their uninvolved skin and evidence of enteropathy. However, of the other twenty-seven patients with a less typical clinical presentation in whom the diagnosis of DH was eventually considered on clinical grounds, there was no difference in the seven without IgA and the twenty with IgA in the uninvolved skin.

In this study, histological examination of involved skin did not appear to be particularly helpful in establishing the diagnosis. The features usually considered to be characteristic of DH, i.e. subepidermal blisters, infiltration with eosinophils and papillary micro-abscesses, were present in only five of the thirty patients who had biopsies. All these five patients did in fact have IgA in the uninvolved skin and an enteropathy. Of the other twenty-five biopsies, twenty-three showed only one or two of the above histological features. All seven patients without IgA in the skin had skin biopsies taken, and the incidence of features suggesting DH was the same as in the eighteen patients with IgA deposits in their skin. In addition, it must be pointed out that the features which are characteristic of DH, namely subepidermal blisters, eosinophils and papillary micro-abscesses, are not diagnostic of DH. Following the observations of Pierard & Whimster (1961) it had been thought that papillary micro-abscesses were diagnostic, but they have since been found in other conditions, including pemphigoid (Connor, Marks & Wilson Jones, 1972).

Following the reports of Cornbleet (1951), Calnan (1954) and Alexander (1955) advocating the efficacy of sulphones, particularly dapsone, in the treatment of DH, therapeutic trials of these drugs have come to be used as a means of establishing the diagnosis of DH. In fact, this is probably the most widely used and accepted criterion for making the diagnosis at the present time. In this series, whilst all thirty-five patients with IgA deposits in their uninvolved skin had their rash controlled with dapsone, so did six of the seven patients without IgA. There are now several reports of skin diseases other than DH responding to dapsone. These include amongst others, vasculitis (Wells, 1969; Thompson *et al.*, 1973), erythema elevatum diutinum (Johnson, 1964; Vollum, 1968; Cream, Levene & Calnan, 1971), pemphigus (Floden & Gentele, 1955; Winkelman & Roth, 1960; Seah *et al.*, 1973b), papular urticaria (Hewitt, Walton & Waterhouse, 1971), and subcorneal pustular dermatosis (Sneddon & Wilkinson, 1956). The mechanism of action of dapsone in DH is unknown, and although it certainly does control the rash, the finding that it suppresses or improves other dermatoses indicates that therapeutic trials in DH are not specific in establishing the diagnosis. It is interesting to note that one patient whose rash was controlled by dapsone or sulphapyridine for 11 years was eventually shown to have pemphigus (Seah *et al.*, 1973b). In addition, three of the remaining six patients without IgA in the uninvolved skin, whose rashes were initially dapsone responsive, now no longer require dapsone and are free of their eruptions. In contrast, none of the thirty-five patients with IgA in their skin have ever shown such spontaneous remissions.

A final appraisal of the diagnostic criteria evaluated here indicates that of the forty-two patients studied, seven are considered by us not to have DH. None of these seven patients had IgA in their uninvolved skin. This is in contrast to the remaining thirty-five, all of whom had IgA deposits. Of these thirty-five patients, thirty-four had evidence of a small bowel enteropathy. It would appear that at the present time, the presence of IgA in the uninvolved skin is the simplest and most reliable way of establishing the diagnosis of DH. We believe that for a GFD to be effective in DH, it must be strict, and at the present time, life-long. Therefore, before such a diet is prescribed, the diagnosis of DH should be established by the finding of IgA in the uninvolved skin and an invariably increased lymphocytic infiltration in the small intestinal epithelium indicating a gluten-sensitive enteropathy.

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Antireticulin antibody: Incidence and diagnostic significance

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Antireticulin antibody: Incidence and diagnostic significance

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SUMMARY Sera from 101 patients with adult coeliac disease, 46 patients with childhood coeliac disease, 50 patients with dermatitis herpetiformis, and 479 patients with various other diseases, including skin, gastrointestinal, haematological, and immunological disorders, have been tested for the presence of the antireticulin antibody. Positive sera were retested at higher dilutions. Antireticulin antibody was only found in a significant proportion of patients with three diseases, ie, coeliac disease, dermatitis herpetiformis, and Crohn's disease. Antireticulin antibody was present in 38 out of 101 patients (38%) with adult coeliac disease, 27 out of 46 patients (59%) with childhood coeliac disease, 11 out of 50 patients (22%) with dermatitis herpetiformis, and nine out of 38 patients (24%) with Crohn's disease. In the 434 other patients with various disorders the antireticulin antibody was present in only six (1·4%) (two patients were pregnant, one had vitiligo, one had tropical sprue, one had reticulum cell sarcoma, and one had pernicious anaemia). In patients with gluten-sensitive enteropathy, ie, coeliac disease and dermatitis herpetiformis, there was a significantly higher incidence in patients taking a normal diet compared with those on a gluten-free diet. The presence of antireticulin antibody would appear to be particularly helpful in diagnosing childhood coeliac disease as it was found in 22 out of 26 patients (85%) taking a normal diet.

An antibody to reticulin was first described in patients with dermatitis herpetiformis and adult coeliac disease by Seah, Fry, Hoffbrand, and Holborow (1971a) and subsequently in childhood coeliac disease (Seah, Fry, Rossiter, Hoffbrand, and Holborow, 1971b). The presence of antireticulin antibody in coeliac disease has been confirmed by Alp and Wright (1971) and by Von Essen, Savilahti, and Pelkonen (1972). Alp and Wright (1971) also detected the antibody in a proportion of patients with Crohn's disease and occasionally in patients with other diseases.

The significance of this antibody is, therefore, uncertain. It is possible, however, that its presence could be a useful test for gluten-sensitive enteropathy as occurs in patients with dermatitis herpetiformis and coeliac disease, and to investigate this possibility we have compared the incidence of the antibody in these conditions and in other gastrointestinal, haematological, skin, and immunological disorders.

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Patients and Methods

PATIENTS

Sera from the following groups of patients were studied.

Adult coeliac disease

One hundred and one patients, of whom 50 were taking a gluten-free diet and 51 a normal diet.

Childhood coeliac disease

Forty-six patients, of whom 20 were taking a gluten-free diet and 26 were receiving gluten.

Dermatitis herpetiformis

Fifty patients, of whom 20 were taking a gluten-free diet and 30 a normal diet.

In addition, patients with the following gastrointestinal disorders were studied: other malabsorption states 35; Crohn's disease 38; ulcerative colitis 11; tropical sprue 52; children with a variable degree of jejunal villous atrophy, thought not to be

gluten induced (eg, after gastroenteritis malabsorption) 13; alpha-chain disease 4.

Patients with the following skin disorders were also studied: pemphigus 14; pemphigoid 15; vitiligo 15; psoriasis 20; cutaneous vasculitis 8; other skin disorders (including erythema nodosum, erythema multiforme, eczema, photosensitivity, drug eruptions, urticaria, necrobiosis lipoidica, and lichen sclerosis) 36.

The following haematological disorders were studied: adult pernicious anaemia 40; nutritional megaloblastic anaemia 10; autoimmune haemolytic anaemia 25; myelosclerosis 5; chronic lymphocytic leukaemia 9.

Patients with the following conditions in which immune disturbances may occur were also studied: sarcoidosis 12; pregnancy 17; systemic lupus erythematosus 7; alopecia areata 4; myaesthenia gravis 4; thyrotoxicosis 1; scleroderma 6; rheumatoid arthritis 15; fibrosing alveolitis 1; lipodystrophy 1.

Finally, a miscellaneous group of seven patients was studied suffering from rickets 2; reticulum cell sarcoma 1; tuberculosis 1; peptic ulcer 1; leg ulcer 1; Parkinson's disease 1.

METHODS

Sera from all patients were stored at −20°C until tested. The presence of antireticulin antibody was detected by indirect immunofluorescence as described previously (Seah *et al*, 1971a). Sera were tested without prior knowledge of the patients' diagnoses and were performed in batches with known positive and negative antireticulin antibody sera acting as controls. All sera were screened at an initial dilution of 1:10 and positive sera were subsequently retested at dilutions of 1:20, 1:40, and 1:80. Some sera still gave strong immunofluorescent staining at a 1:80 dilution and could obviously have been titrated further but it was decided to take 1:80 as the upper limit for the purpose of this study. The endpoint of the titration was taken when weak but definite immunofluorescent staining was seen. Wellcome reagents fluorescein (FITC)-labelled sheep-anti-human immunoglobulin in a dilution of 1:32 was used throughout the study. Prior testing of the conjugate by gel diffusion and chessboard titrations against known immunoglobulin class antinuclear antibodies confirmed its potency and ability to react with the three main human immunoglobulin classes (IgG, IgM, and IgA). Specimens were mounted in buffered glycerol (pH 8) and examined on a Reichert Diapan microscope with a quartz-iodine light source, a Balzer FITC-3 interference primary filter, and a Wratten 12 secondary filter.

Results

ADULT COELIAC DISEASE

Antireticulin antibody was found to be present in the sera of 38 (38%) of the 101 patients. It was found in 30 (59%) of 51 patients taking a normal diet but in only eight (16%) of 50 taking a gluten-free diet (table I). Of the patients taking a gluten-free diet, the antibody was found at dilutions of 1:10 in five, 1:20 in two, and 1:40 in one. Of the 30 patients on a normal diet the antibody was present in dilutions of 1:10 in 21, 1:20 in 2, 1:40 in five, and 1:80 in two (see fig).

CHILDHOOD COELIAC DISEASE

Antireticulin antibody was present in 27 (59%) of the 46 patients. It was present in 22 (85%) of 26 patients taking a normal diet and in five (25%) of 20 taking a gluten-free diet (table I). Of the patients taking a normal diet the antibody was present in a titre of 1:10 in four, 1:20 in three, 1:40 in four, and 1:80 in 11. Of the five patients taking a gluten-free diet the antibody was present at a titre of 1:10 in two, 1:20 in one, and 1:80 in two (see fig).

DERMATITIS HERPETIFORMIS

Antireticulin antibody was found in 11 (22%) of the 50 patients. It was only found in two (10%) of the

Group	*Total Studied*	*Dietary Status*		*Antireticulin Antibody Positive*		*Total Antireticulin Antibody Positive*
		On Gluten-free Diet	*On Normal Diet*	*On Gluten-free Diet*	*On Normal Diet*	
Childhood coeliac disease	46	20	26	5 (29%)	22 (85%)	27 (59%)
Adult coeliac disease	101	50	51	8 (16%)	30 (60%)	38 (38%)
Dermatitis herpetiformis	50	20	30	2 (10%)	9 (30%)	11 (22%)

Table I *Incidence of antireticulin antibody and relationship to a gluten-free diet in childhood and adult coeliac disease and dermatitis herpetiformis*

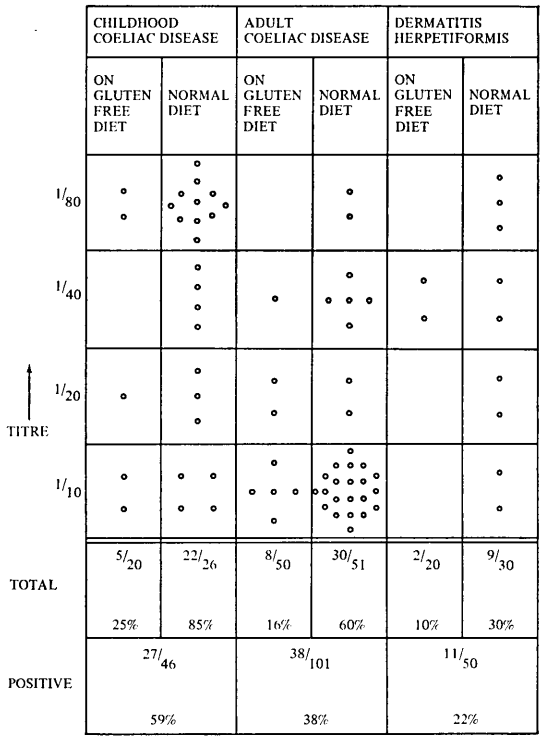

Fig. *Antireticulin antibody related to diet in childhood and adult coeliac disease and dermatitis herpetiformis.*

20 patients taking a gluten-free diet but in nine (30%) of the 30 taking a normal diet (table I). In the two patients taking a gluten-free diet the antibody was found at a titre of 1:40. In the nine patients on a normal diet the antibody was present at a titre of 1:10 in two, 1:20 in two, 1:40 in two, and 1:80 in three (see fig).

CROHN'S DISEASE

This was the only other disease in which the antireticulin antibody was found in a significant proportion of patients since it was present in nine (24%) of the 38 patients. The antibody was only found at the lower titres, however, ie, 1:10 in seven and 1:20 in two. It was not present at 1:40 or 1:80 in any of these patients.

OTHER DISORDERS

Of the 434 other sera tested, the antireticulin antibody was present in six (1·4%). It was found in two patients who were pregnant, in one patient with tropical sprue, in one patient with pernicious anaemia, in one patient with a reticulum cell sarcoma, and in one patient with vitiligo (table II). It was only present at a titre above 1:10 in the patient with reticulum cell sarcoma (titre 1:20) and in the patient with vitiligo (1:80).

Gastro-intestinal Disorder	*No.*	*Anti-reticulin Antibody Positive*	*Haemato-logical Disorder*	*No.*	*Anti-reticulin Antibody Positive*	*Skin Disorder*	*No.*	*Anti-reticulin Antibody Positive*	*Immune Disturbance*	*No.*	*Anti-reticulin Antibody Positive*	*Miscellaneous Disorders*	*No.*	*Anti-reticulin Antibody Positive*
Crohn's disease	38	9	Adult pernicious anaemia	40	1	Pemphigus	14	0	Sarcoidosis	12	0	Rickets	2	0
Tropical sprue	52	1	Nutritional megoblastic anaemia	10	0	Pemphigoid	15	0	Pregnancy	17	2	Reticulum cell sarcoma	1	1
Ulcerative colitis	11	0	Auto-immune haemolytic anaemia	10	0	Vitiligo	15	1	Systemic lupus erythema-tosus	7	0	Tuberculosis	1	0
Other malabsorp-tion states	35	0	Congenital haemolytic anaemia	25	0	Psoriasis	20	0	Alopecia areata	4	0	Peptic ulcer	1	0
Failure to thrive with gastro-intestinal symptoms	13	0	Chronic lymphocytic leukaemia	9	0	Cutaneous vasculitis	8	0	Rheumatoid arthritis	15	0	Parkinson's disease	1	0
Alpha-chain disease	4	0	Myelo-sclerosis	5	0	Miscel-laneous group	36	0	Scleroderma	6	0	Leg ulcer	1	0
									Myasthenia gravis	4	0			
									Miscel-laneous	3	0			

Table II *Incidence of antireticulin antibody in other diseases*

Discussion

These results show that the antireticulin antibody occurs predominantly in three diseases: adult and childhood coeliac disease, dermatitis herpetiformis, and other skin, gastrointestinal and haematological disorders, which may have similar clinical presentations did not have the antibody present in their sera. The incidence of the antireticulin antibody in this study gives similar results to those previously reported, ie, approximately a third of patients with adult coeliac disease (Seah *et al*, 1971a; Alp and Wright, 1971), two-thirds of patients with childhood coeliac disease (Seah *et al*, 1971b; Alp and Wright, 1971; Von Essen, *et al* 1972), a fifth of patients with dermatitis herpetiformis (Seah *et al*, 1971a), and a quarter of patients with Crohn's disease (Alp and Wright, 1971). In addition to the higher incidence of the antibody in childhood coeliac disease compared to adult coeliac disease and dermatitis herpetiformis there was a much higher incidence of the antibody at a higher titre in the childhood form of the disease. Children with coeliac disease more commonly respond to gluten withdrawal than adults and the particularly high incidence of the antibody in childhood coeliac disease may be related to this high incidence of gluten sensitivity. This is consistent with the finding of a definite relationship in coeliac disease and dermatitis herpetiformis between the presence of antireticulin antibody and whether or not the patients were taking gluten in the diet since all three groups—childhood coeliac disease, adult coeliac disease, and dermatitis herpetiformis—showed a significantly lower incidence of the antibody in the sera if the patients were taking a gluten-free diet.

At the present time, the exact significance of antireticulin antibody is uncertain and if it is due to cross reactivity between gluten and reticulin it is difficult to explain why the antibody is not present in the sera of all untreated patients who have dermatitis herpetiformis or coeliac disease, ie, all patients with gluten-sensitive enteropathy. It is possible, however, that all the antibody is bound to tissues in some patients and this could account for its absence from serum. Moreover, many of the patients classified as taking a normal diet had, in fact, received a gluten-free diet at some time previously and may still be taking relatively small amounts of gluten. On the other hand, the antibody in the sera of some patients receiving a gluten-free diet may arise because they are not strictly adhering to the diet.

We found no relation between the degree of histological abnormality of the small intestine in coeliac disease and dermatitis herpetiformis and the presence of antireticulin antibody. Von Essen *et al* (1972) did not detect antireticulin antibody in the sera of children with cow's milk intolerance, 10 of whom had been exposed to gluten, although the histological features of the small intestinal mucosa were similar to those seen in childhood coeliac disease. Moreover, these workers also found the antibody in four of 23 patients with IgA deficiency who showed normal jejunal mucosa. Further, as shown here, the antibody is absent from the sera of patients with the diffuse small intestinal mucosal damage of tropical sprue. Taken altogether, these findings, therefore, do not support the suggestion of Alp and Wright (1971) that the antireticulin antibody may be a non-specific result of dietary protein passing through a damaged small intestinal mucosa.

The finding of antireticulin antibody in approximately 25% of patients with Crohn's disease is difficult to explain if the antibody is closely related to gluten sensitivity. None of the positive sera in the patients with Crohn's disease studied here, however, showed antibody titres as high as those found in some cases of untreated childhood coeliac disease, adult coeliac disease, and dermatitis herpetiformis. Whether the antibody present is identical in all these conditions and why it arises in both gluten-sensitive enteropathy and Crohn's disease remains obscure.

The finding of antireticulin antibody in six out of 434 patients with various disorders (two were pregnant, one had vitiligo, one pernicious anaemia, one tropical sprue, and one reticulum cell sarcoma) has to be explained. Subclinical coeliac disease is not rare and it is possible that these six patients have a gluten-sensitive enteropathy which has not been diagnosed but it was not possible to obtain jejunal biopsy material from any of them. Only one of the patients had the antibody present at a titre of 1:80 and this was the patient with vitiligo. This patient was under weight and there was a family history of vitiligo suggesting autoimmune disease. Another possibility, and this, as mentioned above, may apply to patients with Crohn's disease, is that there is, in fact, a difference in the antireticulin antibody between patients who have gluten sensitivity and those who do not. Further work is in progress to investigate this theory.

Our findings do not support those of Alp and Wright who found the antireticulin antibody in ulcerative colitis and psoriasis. These authors also found the antibody in patients with uveitis, aphthous ulceration, and the irritable colon syndrome, and in as many as three (4%) of 68 normal adults, whereas we have not detected the antibody in 60 normal adult subjects. The difference in these findings may arise because Alp and Wright tested their sera at a dilution of 1:5 whereas the highest concentration we have used is 1:10.

At the present time it would appear that the presence of the antireticulin antibody and its titre, as demonstrated by indirect immunofluorescence, is a useful test in establishing the diagnosis of coeliac disease, dermatitis herpetiformis, and Crohn's disease. It would seem to be of particular help in the diagnosis of coeliac disease in children as there is a particularly high incidence of the antibody in this disease and because Crohn's disease is rare in children.

We are grateful to various colleagues for contributing sera, to Dr N. Maldonado (Chief of Haematology, University Hospital, San Juan, Puerto Rico) for providing sera from patients with tropical sprue and to Mr S. Kumar for expert technical assistance.

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Clinical and Laboratory Investigations

Ultrastructural observations on uninvolved skin in dermatitis herpetiformis

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SUMMARY

Clinically normal skin from patients being treated for dermatitis herpetiformis was examined with the electron microscope.

Cytoplasmic processes from the basal epidermal cells penetrated the dermis through discontinuities in the basal lamina which otherwise was normal in structure and thickness. The fibrous network of the dermis consisting of collagen, small reticular fibrils, anchoring fibrils and elastin, appeared normal.

In patients receiving only dapsone, membrane-bound 'vacuoles' were found just below the basal lamina. They contained a fibrillar material of low electron density and were often associated with cell processes or complete cells, the classification of which was difficult. It is suggested that these vacuoles may be implicated in the reaction between reticulin and immunological complexes.

In two specimens, early blister formation was also seen in the dermal papillae. The basal lamina remained applied to the stratum basale and was only absent where basal cell processes projected into the blister space which contained fibrin deposits. Vesiculated structures observed in the region of the blisters are interpreted as abnormal sensory nerve endings.

This evidence supports the view that disruption of the basal lamina and blister formation in dermatitis herpetiformis are secondary to an earlier reaction.

Light microscope observations have established that in dermatitis herpetiformis, the skin lesions are situated at or below the dermal–epidermal junction of the dermal papillae (Pierard & Whimster, 1961). In the formation of the typical blister characterizing these lesions, the epidermis is separated from the dermis and examination with the electron microscope has revealed that in the region of the separation the basal lamina is disrupted or absent (Bellone & Caputo, 1966; Pierard & Kint, 1968; Fry & Johnson, 1969; Jakubowicz, Dabrowski & Maciejewski, 1970; Rodrigo, 1972); from this evidence it has been suggested that the basal lamina is primarily involved with the disease process (Fry & Johnson, 1969; Jakubowicz *et al.*, 1970). Recent investigations on clinically normal skin in dermatitis herpetiformis using immunological techniques have shown that antireticulin antibodies are present beneath, but probably not involving, the basal lamina of the dermal papillae (Seah *et al.*, 1972). Therefore, it was felt necessary to examine the dermal–epidermal junction of uninvolved skin in dermatitis herpetiformis at the ultrastructural level. This paper describes changes which have been observed in the dermis of clinically normal skin from patients with dermatitis herpetiformis.

MATERIALS AND METHODS

Specimens were taken from a series of twelve patients with dermatitis herpetiformis in whom the rash was being treated with dapsone and/or a gluten-free diet. Five patients were on dapsone alone, three were solely on a gluten-free diet and four were on both dapsone and a gluten-free diet. All skin areas chosen were clinically normal. Eleven biopsies were taken from the gluteal region and one from the forearm; they were performed under 2% lignocaine anaesthesia. The tissue was fixed in 4% buffered glutaraldehyde and immediately post-fixed in 1% buffered osmium tetroxide. After dehydration with a graded series of ethyl alcohols, the specimens were embedded in Taab resin. Thick sections, stained with toluidine blue, were examined to aid orientation of the tissue; subsequently thin sections were cut, stained with uranyl acetate and/or lead citrate (Reynolds, 1963) and examined on a Siemens Elmiskop I electron microscope.

RESULTS

In general, the structure of the basal cells of the epidermis was normal, but in some specimens cytoplasmic processes from the dermal surface of basal cells were seen passing through discontinuities in the basal lamina to penetrate the dermis for short distances (Fig. 1). These processes were usually smaller than those described in the region of a blister (Fry & Johnson, 1969), but they were similar

FIGURE 1. The dermal–epidermal junction showing vacuoles (v) in the dermis close to a basal cell process (p) which projects through a discontinuity in the basal lamina (thick arrows). Note the normal hemidesmosomes (h) and reticular fibrils (r) (×20,800).

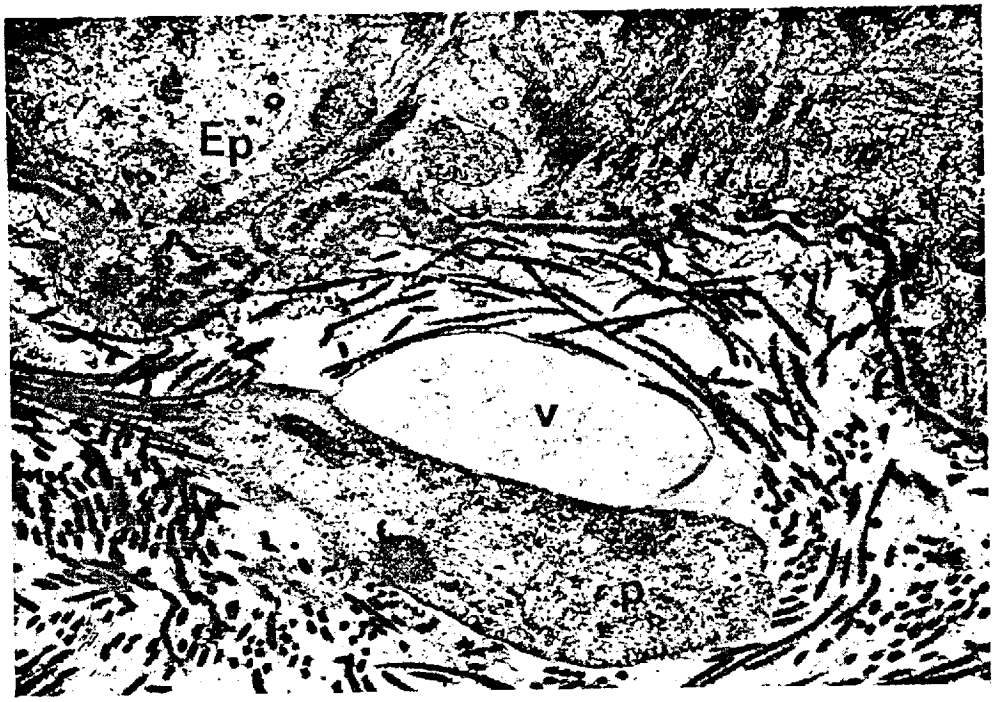

FIGURE 2. A membrane-bound vacuole (v) filled with a fibrillar material and associated with a cell process (p). Ep, epidermis (×20,000).

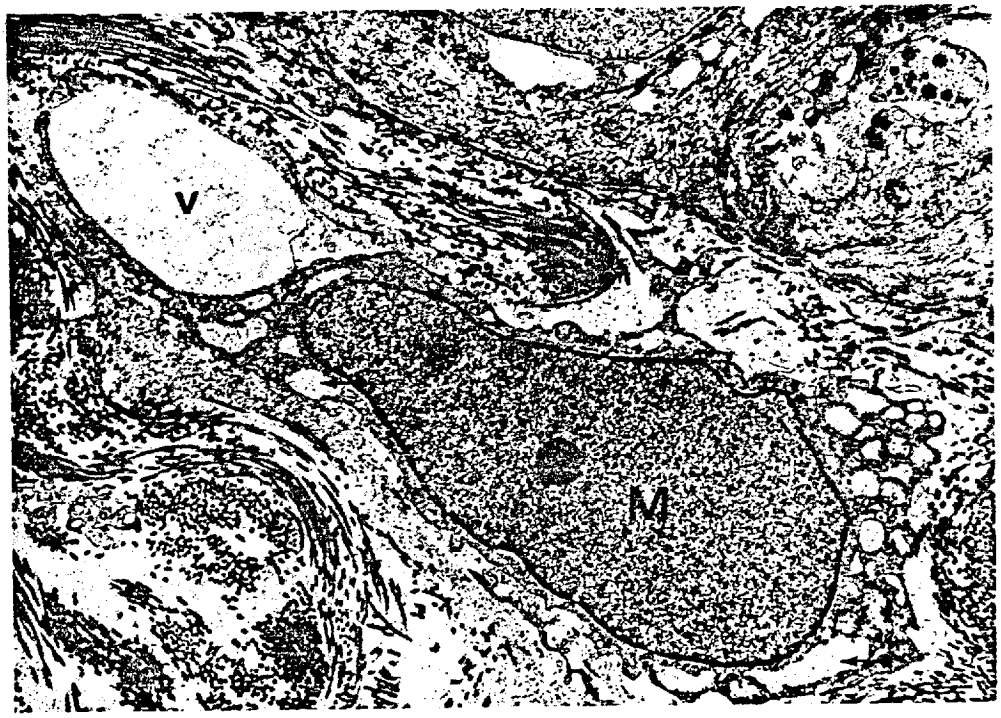

FIGURE 3. A vacuole (v) partly surrounded by a process from a dermal cell (M) which contains numerous vesicles (arrows) (×7500).

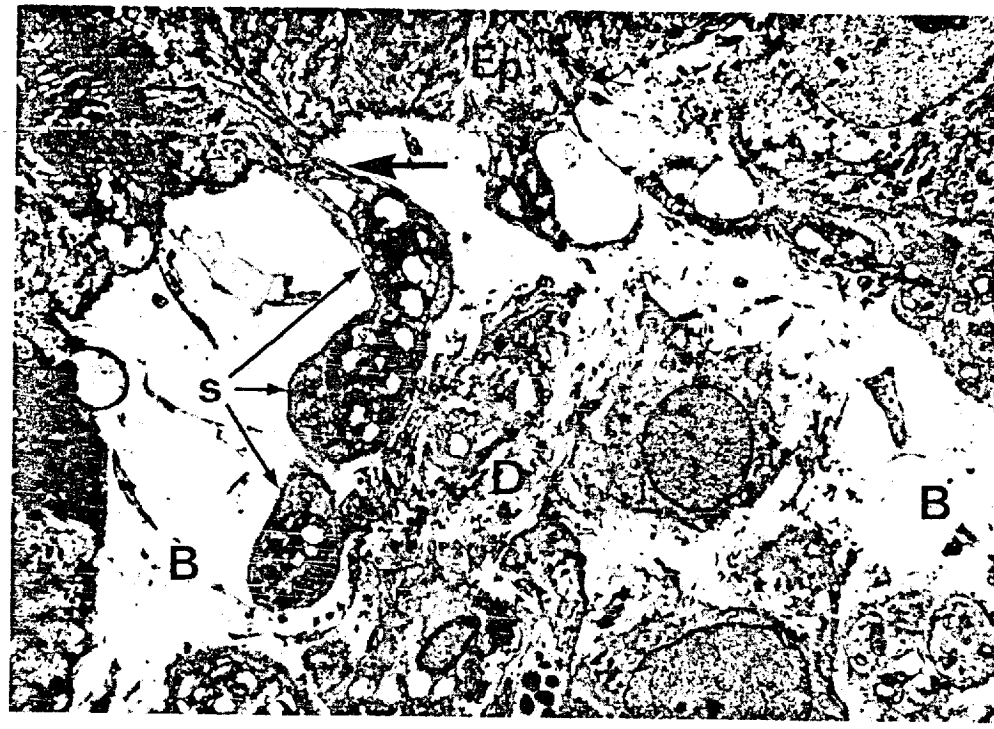

FIGURE 4. Early blister formation with separation of the epidermis (Ep) from the dermis (D) to produce a blister space (B). Note the vesiculated structures (s), one of which is associated with a basal cell process (thick arrow) (×4000).

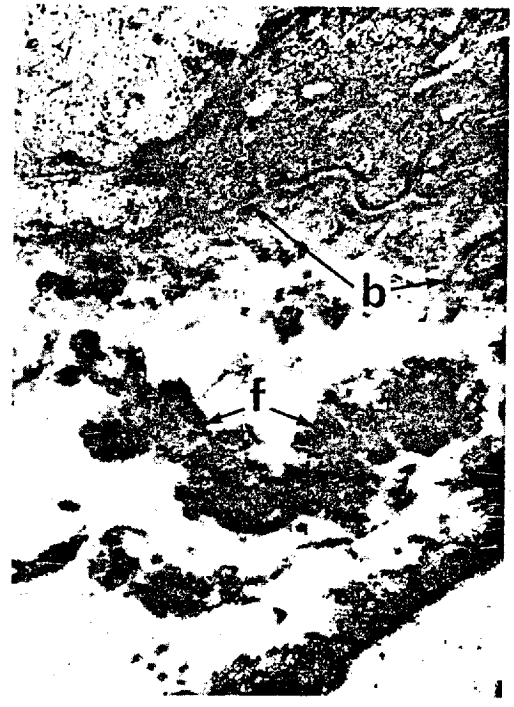

FIGURE 5. Fibrin deposits (f) close to the epidermal basal lamina (b) which is intact (×20,000).

in that they did not contain cell organelles or tonofilaments. There was a normal arrangement of hemidesmosomes along the cell membrane bordering the dermis where the basal lamina was present (Fig. 1). The cytoplasm of the basal cells contained normal amounts of tonofilaments and the mitochondria and other cell organelles were normal in structure and distribution. Small deposits of glycogen were sometimes present.

The basal lamina appeared normal both in thickness and structure and, apart from occasional gaps where the basal cell processes penetrated the dermis (Fig. 1), it formed a continuous layer between the epidermis and dermis. Anchoring fibrils were present along its dermal surface.

The fibrous network of the dermis consisted of numerous collagen bundles interspersed by clumps of elastin; both these components were normal in appearance and distribution. Among the collagen fibres and subjacent to the basal lamina, groups of smaller 'reticular' fibrils were seen running for variable distances through the dermis (Fig. 1). Occasionally these fibrils formed clumps either close to the basal lamina or lying deeper in the dermis.

In all of the specimens taken from patients who were solely on dapsone therapy and in one from a patient on dapsone and a gluten-free diet, vacuole-like spaces were seen in the dermis just below the epidermal basal lamina and associated anchoring fibrils (Figs. 1–3). They were usually situated in the dermal papillae and in many cases they were found in the vicinity of the basal cell processes projecting into the dermis (Fig. 1). The 'vacuoles' were surrounded by a membrane which, in places, became indistinct and they contained a fibrillar material of low electron density. Sometimes a cell process (Fig. 2) or a complete cell surrounded part of the vacuole (Fig. 3); in all cases the membrane bounding the vacuole remained separate from that of the cell. In many respects these cells did not appear to be particularly well differentiated and consequently they were difficult to classify. Most of them had branching cell processes while in some cells the cytoplasm contained vesicles in which there was a material of low electron density (Fig. 3).

Specimens from two patients solely on dapsone had in addition to the vacuoles, evidence of early blister formation in some of the dermal papillae. In the region of such an early blister, most of the dermis was separated from the epidermis (Fig. 4), leaving the basal lamina and groups of fibrils still applied to the dermal surface of the stratum basale. The basal lamina was absent where processes from the basal epidermal cells projected into the blister space; these processes were longer than those which were observed in relation to the vacuoles. No actual thickening of the basal lamina was seen, but subjacent to it and within the blister space deposits of material resembling fibrin were present (Fig. 5). In the dermis beneath the blister space, there were several vesiculated structures (Figs. 4 and 6) and these were surrounded by cell processes, around the outer parts of which a basal lamina could be distinguished (Fig. 6). Some of the vesicles contained membranes which resembled the cristae of mitochondria (Fig. 6). Vesiculated structures were seen in contact with dermal processes of the basal cells (Fig. 4), but it was not possible to demonstrate the membranes clearly enough to observe the exact nature of the junction. Among the dermal elements beneath the blister, Schwann cell processes with unmyelinated nerve axons were also present.

Other components of the dermis were relatively normal. Macrophages and leukocytes were occasionally found, but rarely eosinophils. The capillaries showed no evidence of abnormality either in size or structure.

DISCUSSION

Previous ultrastructural studies on dermatitis herpetiformis have concentrated on the blister stage of the lesion when it is difficult to distinguish between the primary effects of the disease process and the non-specific secondary reaction. Rodrigo (1972) included in his material specimens of uninvolved

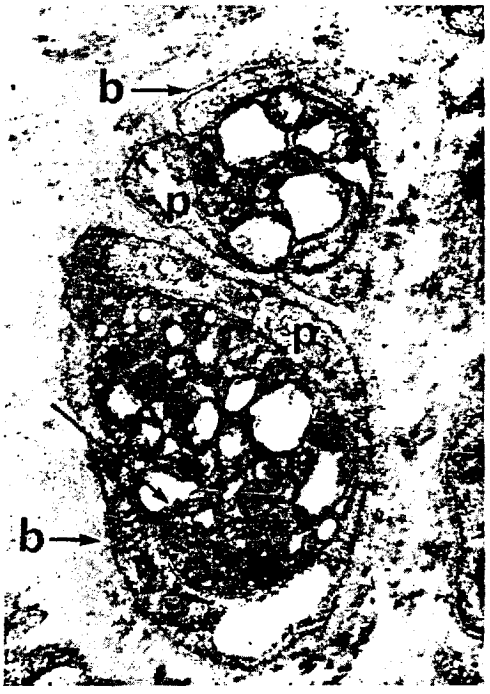

FIGURE 6. A higher magnification of two vesiculated structures which consist of a cell process (p) surrounded by a basal lamina (b) and enclosing numerous vesicles some of which have membranes resembling the cristae of mitochondria (arrow) (× 15,000).

skin from patients with blisters, but states that abnormalities in its structure were slight and inconstant. In the early blister, most investigators report that the basal lamina is disrupted or absent (Bellone & Caputo, 1966; Pierard & Kint, 1968; Fry & Johnson, 1969; Jakubowicz *et al.*, 1970; Rodrigo, 1972) and a fibrin-like material is deposited in the blister space (Jakubowicz *et al.*, 1970, 1971; Rodrigo, 1972). Although all the specimens in the present study were taken from clinically normal skin, two showed evidence of blister formation at the microscopic level. In these cases, the basal lamina appeared normal apart from breaks through which basal cell processes penetrated the dermis and the hemidesmosomes present did not exhibit the rarefaction which has been described in early lesions (Rodrigo, 1972). While the deposition of fibrin may be a characteristic of dermatitis herpetiformis (Jakubowicz *et al.*, 1971; Mustakallio, Blomqvist & Laiho, 1970), it is felt that this is probably secondary to an earlier reaction. Recent work on the experimental production of blisters has shown that a coagulum of fibrin and remains of basal lamina form a surface over which the regrowth of epidermal cells can occur (Krawczyk, 1971).

The vesiculated structures observed beneath the early blisters resemble in some respects the profiles described as sensory nerve endings (Burnstock & Iwayama, 1971) where the axon is packed with small, oval mitochondria. If the vesicles represent mitochondria, they are swollen and their cristae are disrupted. The presence of such apparently abnormal nerve endings in relation to the early lesion is interesting in view of the pruritus which is associated with the clinical pre-blister stage of the disease.

The fibrous network of the dermis contained in addition to collagen fibres, numerous smaller fibrils which correspond to the 'reticular' fibrils described in normal skin (Berger & Hundeiker, 1967). Whether these fibrils correspond to the tissue which is described as reticulin at the light microscope

level has yet to be demonstrated, but they may be the structures onto which the 'antireticulin' antibodies described by Seah *et al.* (1972) are attached. There are certainly a number of such fibrils close to the basal lamina in the region where the antibodies are demonstrated.

The most obvious abnormal finding in clinically normal skin from patients solely on dapsone therapy was the presence of membrane-bound vacuoles in the dermis. The nature of the fibrillar material they contain cannot be resolved on the present evidence, but it could represent a collection of fluid induced by a local concentration of protein such as antibodies. Other possibilities include collections of ground substance or even cytoplasmic processes from epidermal cells similar to those seen in healing skin (Odland & Ross, 1968). However, the latter suggestion is unlikely as the vacuoles do not resemble the basal cell processes observed in the present investigation. Confluence of several vacuoles could well be the initial stage in the formation of the microscopic blisters previously discussed. Except for one specimen, the vacuoles were not seen in patients who were on a gluten-free diet with or without dapsone which raises the possibility that the presence of gluten in the diet of patients solely on dapsone therapy may still allow the pathological process to continue at a sub-clinical level. A more detailed study of the present findings in relation to treatment is in progress.

Owing to the difficulty in cell classification, the exact relationship of the dermal cells and the cell processes to the vacuoles could not be determined. The most probable explanation, however, is that the cells are some type of macrophage which are attempting to phagocytose the content of the vacuole. Likewise, the association between the dermal processes of the basal cells and the vacuoles is difficult to explain. Defects in the basal lamina, with dermal processes from basal cells, are rarely encountered in skin from normal adults (Riches, personal observations), but they have been observed in other skin diseases (Sugár, 1968; Cox, 1969). Cox (1969) suggests that basal lamina defects are in some way associated with cellular proliferation in the epidermis. Although this may explain the findings in psoriasis and other proliferative disorders, it is difficult to apply such an hypothesis to dermatitis herpetiformis where the cell turnover is probably normal. An alternative reason for dermal processes from basal cells in dermatitis herpetiformis could be that the basal cells are in some way secondarily implicated in the pathological process.

The findings of the present study are compatible with the results of Seah *et al.* (1972) who suggest that the primary site of the pathology in dermatitis herpetiformis is the reticulin fibres of the dermis which cross-react with immunological complexes. The changes in the basal lamina could be secondary to this reaction, and possibly due to local mechanical disruption; while the development of the blister with deposition of fibrin is consistent with an immune-complex disease.

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Splenic Atrophy in Dermatitis Herpetiformis

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British Medical Journal, 1972, 2, 438–440

Summary

Twenty-four patients with dermatitis herpetiformis were investigated for splenic atrophy by splenic scan and clearance of ^{51}Cr-labelled heat-damaged red blood cells. Eight of them had definite splenic atrophy. The average splenic cross-sectional area of the remaining 16 with normal clearance times was substantially smaller than normal, suggesting some degree of splenic atrophy. No relationship of splenic hypofunction to intestinal biopsy findings, folate status, reticulin antibody, or treatment with a gluten-free diet or dapsone was evident.

Introduction

Considerable interest has been shown in the recent finding that patients with dermatitis herpetiformis show an enteropathy similar to that of coeliac disease (Marks *et al.*, 1966; Fraser *et al.*, 1967; Fry *et al.*, 1967; Brow *et al.*, 1971). Because they found that both the skin and gut lesions improved when patients with dermatitis herpetiformis received a gluten-free diet, and relapsed on reintroduction of gluten, Fry *et al.* (1969) suggested a direct relation between the skin and small intestine in this disorder.

Seah *et al.* (1971a, 1971b) demonstrated the presence of reticulin antibodies in the sera of a proportion of patients with both dermatitis herpetiformis and coeliac disease. Since the spleen is the largest organized collection of lymphoreticular tissue in the body and the occurrence of splenic atrophy in coeliac disease is well established (Martin and Bell, 1965; McCarthy *et al.*, 1966; Ferguson *et al.*, 1970; Marsh and Stewart, 1970), we have investigated splenic size and function in a group of patients with dermatitis herpetiformis to further clarify the relation between dermatitis herpetiformis and coeliac disease.

Patients and Methods

Twenty-four consecutive patients attending a dermatitis herpetiformis clinic were studied (see Table). There were 14 men aged 28-75 (average 52) and 10 women aged 26-65 (average 38). At the time of these studies seven patients had their rash controlled by a gluten-free diet alone, nine were receiving a gluten-free diet, and dapsone, and eight were receiving dapsone only.

Assessment of Splenic Function.—Autologous red cells were labelled with 150 μCi of ^{51}Cr and damaged by heating for 20 minutes at 49·5°C. Splenic function was assessed by measuring the rate of clearance of the ^{51}Cr-labelled cells. After the intravenous injection of these cells, blood samples were taken at 3, 10, 20, 30, and 60 minutes, haemolysed, and radioactivity counted

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in a well-scintillation counter. The results were plotted on semi-logarithmic graph paper, taking the activity of the three-minute sample as 100%. The half-time of clearance of the ^{51}Cr label from the circulation (read from the graph) was used as an index of splenic function. In control patients suffering from localized head and neck malignancy or bladder carcinoma, Marsh *et al.* (1966) found this time to be within the range of 10-16 minutes. Subsequent studies in our laboratory on 20 other patients who have had no evidence of splenic disease have shown half-clearance times within this range. One hour after the injection of the labelled, damaged cells the patient's splenic area was scanned with a dual-detector scanner (Picker). The cross-sectioned area of the spleen outlined on this antero-posterior scan was calculated with a planimeter.

Haematological Investigations.—Routine blood counts were measured on the day of the splenic study (Dacie and Lewis, 1968). Two peripheral blood films from each patient were stained by a May-Grünwald-Giemsa technique and examined; the presence of Howell-Jolly bodies with or without target cells and of crenated and contracted red cells was considered presumptive evidence of splenic atrophy. Serum folate levels were determined by microbiological assay with *Lactobacillus casei* as test organism (Waters and Mollin, 1961). The normal range is 6-21 ng/ml. Red cell folate was measured as described by Hoffbrand *et al.* (1966). The normal range is 160-640 ng/ml packed cells.

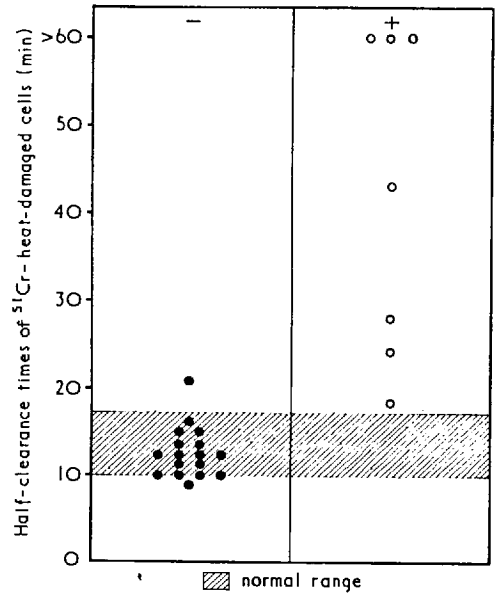
Small-intestinal Biopsy.—Biopsy specimens were taken from the upper jejunum at the ligament of Treitz by using a special capsule (Crosby and Kugler, 1957) under radiological control. The specimen was fixed in 10% formol-saline before processing for histological examination and it was examined and photographed with a Zeiss standard Universal dissection microscope using a 3-in (7·5-cm) objective. Sections of the specimen were then cut and stained: (1) with haematoxylin and eosin and (2) according to the methylgreen-pyronin technique for the specific identification of plasma cells.

Reticulin Antibody.—This was detected as described by Seah *et al.* (1971a).

Results

Clearance of Heat-damaged Red Cells and Splenic Cross-sectional Area.—Eight of the 24 patients had prolonged half-clearance times (see Table). Three patients (Cases 1, 2, and 3) who had the most prolonged half-clearance times, of greater than one hour, had no evidence of functioning splenic tissue on the scintillation scan. In the remaining five patients with abnormal clearance times, three (Cases 4, 5, and 6) had particularly small splenic cross-sectional areas of 18, 13, and 14 cm^2 respectively. Of the 16 patients with normal clearance times (Cases 9-24), one (Case 14) had a splenic cross-sectional area which could be considered to be definitely smaller than normal (16 cm^2). However, the mean area among these 16 patients was only 42·0 cm^2, compared with the Larson *et al.* (1970) mean normal level of 52·8 cm^2, and as many as 14 of the 16 patients had splenic cross-sectional areas of less than 52·8 cm^2, as did Cases 7 and 8 who had slow clearance times.

Blood Film Evidence of Splenic Atrophy.—Howell-Jolly bodies were obvious in the films of 7 of the 24 patients. The amount of targetting, contraction, and crenation of red cells in these patients was variable, and minor degrees of these latter red cell changes were observed in a proportion of the patients in whom no Howell-Jolly bodies were observed. All the patients with Howell-Jolly bodies had longer than normal half-clearance times, but one (Case 7), who had a slow half-clearance time (19 minutes), showed no blood film evidence of splenic atrophy (see Chart).



Comparing the half-clearance time of ^{51}Cr heat-damaged red cells with the peripheral blood film in the 24 patients with dermatitis herpetiformis. Closed circles = *no* Howell-Jolly bodies; open circles = Howell-Jolly bodies present.

Splenic Studies in Dermatitis Herpetiformis

Case No.	Sex and Age	Half-Clearance of Damaged Cells (Min)	Scan Size (cm^2)	Blood Film Evidence of Splenic Atrophy	Gut Biopsy*			Therapy	Folate Level in Serum/R.B.C. (ng/ml)	Reticulin Antibody
					Macroscopic	Epithelium	Cellular Infiltrate†			
1	F. 37	90·0	Nil	+	Flat	Cuboidal	+	G.F.D. + dapsone	3·0/76	+
2	M. 60	85·0	Nil	+	Convoluted	Columnar+cuboidal	+	G.F.D.	4·8/225	—
3	F. 35	75·0	Nil	+	Flat	Columnar+cuboidal	++	G.F.D.+dapsone	2·0/186	—
4	M. 71	42·0	18	+	Flat	Cuboidal	+	G.F.D.	Receiving folic acid	+
5	M. 54	27·0	13	+	Convoluted	Columnar	+	G.F.D.	2·0/108	—
6	M. 74	24·0	14	+	Convoluted	Columnar	++	Dapsone	2·4/197	—
7	F. 25	19·0	30	—	Fingers+leaves	Columnar	++	Dapsone	7·6/351	—
8	F. 48	17·5	40	+	Flat	Cuboidal	+	Dapsone	Receiving folic acid	—
9	F. 29	15·5	39	—	Flat	Cuboidal	+	Dapsone	2·8/266	—
10	F. 65	14·5	45	—	Flat	Cuboidal	++	G.F.D.	3·9/128	—
11	F. 52	14·0	35	—	Fingers+leaves	Columnar	+	Dapsone	5·2/185	—
12	M. 68	13·0	36	—	Leaves	Cuboidal	++	G.F.D.+dapsone	4·1/125	+
13	M. 64	13·0	42	—	Flat	Cuboidal	+	G.F.D.+dapsone	2·8/445	—
14	F. 38	12·0	16	—	Convoluted	Columnar	+	Dapsone	3·0/111	—
15	F. 32	12·0	46	—	Convoluted	Columnar	+	Dapsone	6·0/363	—
16	M. 49	12·0	36	—	Leaves	Columnar	+	Dapsone	7·5/260	—
17	M. 26	12·0	58	—	Leaves	Cuboidal	+	G.F.D.	3·6/258	+
18	M. 55	11·0	50	—	Fingers+Leaves	Columnar	+	G.F.D.+dapsone	4·4/297	—
19	M. 33	11·0	51	—	Flat	Cuboidal	++	G.F.D.+dapsone	1·0/116	—
20	M. 34	10·0	58	—	Fingers+leaves	Columnar	+	G.F.D.+dapsone	4·6/267	—
21	M. 37	10·0	35	—	Flat+convoluted	Cuboidal	++	G.F.D.+dapsone	5·6/246	—
22	F. 30	10·0	40	—	Flat	Cuboidal	++	G.F.D.	4·1/187	—
23	M. 36	10·0	43	—	Fingers+leaves	Columnar	+	G.F.D.	5·2/198	—
24	M. 56	9·5	42	—	Leaves+convoluted	Columnar	+	G.F.D.+dapsone	5·8/189	—

*Appearances refer to biopsy before gluten-free diet.
†+ = Moderate infiltration. + + = Heavy infiltration.
G.F.D. = Gluten-free diet.

Small-intestinal Biopsy Appearances; Reticulin Antibody; Patients' Diet and Folate Status.—The macroscopic appearances of the intestinal biopsy ranged from flat to normal. Histologically all the biopsies showed varying degrees of cellular infiltration. The three patients in whom no spleen could be demonstrated on the scan all showed macroscopic abnormality. However, there was no overall correlation between splenic size and morphological appearances of the small-intestinal mucosa. Likewise, there was no relation of splenic size to whether or not the patient was on a gluten-free diet, to folate status, or to the presence or absence of the reticulin antibody in the serum.

Discussion

These results show that a third of patients with dermatitis herpetiformis had unequivocal evidence of splenic atrophy, as assessed by clearance of damaged red cells, splenic scan, and blood film appearance. Moreover, the splenic scan studies suggest that most of the remaining patients had some degree of splenic atrophy.

The presence of splenic atrophy in patients with primary malabsorption syndromes has been known for some time. McCarthy *et al.* (1966) found evidence of lymphoreticular atrophy in 4 out of 25 patients with "idiopathic steatorrhoea." Three of these patients had extremely small spleens, while the spleen of the fourth patient was normal in size.

Radioisotope-labelled heat-damaged red cells have been widely used to measure the functional size of the spleen, and there seems to be a relation between clearance rates of the labelled cells and the reticuloendothelial function of the spleen (Marsh *et al.*, 1966; Pettit *et al.*, 1971). Marsh and Stewart (1970) found greater than normal clearances of heat-damaged ^{51}Cr-labelled red cells in 14 out of 19 patients with adult coeliac disease and five of these patients showed no localization of radioisotope in the splenic area and had blood film appearances of splenic atrophy.

Fry *et al.* (1967) found Howell-Jolly bodies and target cells in the blood films of 2 out of 12 patients with dermatitis herpetiformis examined. The finding here of slower than normal clearances in 8 of the 24 patients clearly shows that splenic hypofunction is common in patients with dermatitis herpetiformis. Although at first sight it would seem that splenic hypofunction is somewhat less frequent in dermatitis herpetiformis than in coeliac disease, a closer inspection of the results shows that even the dermatitis herpetiformis patients with normal clearance times tended to have smaller functional splenic cross-sectional areas than normals. The normal splenic cross-sctional area as determined by rectilinear scintillation scanning is not well established. Holzback *et al.* (1962) reported a range between 35 and 85 cm^2 in a series of 23 control patients. Larson *et al.* (1970) studied 26 healthy adults and found a mean cross-sectional area of 52·8 cm^2 in the posterior rectilinear spleen scans. In the 16 dermatitis herpetiformis patients reported here who had normal clearance times the mean cross-sectional area was only 42·0 cm^2; 14 of the 16 patients had areas less than the mean normal value quoted above and a patchy distribution of isotope was evident in some cases. This, though not conclusive, strongly suggests that most of the patients, even with normal clearance, have some degree of splenic atrophy.

No overall correlation was found in this study between the size of the spleen and the severity of the small-intestinal mucosal abnormality. In addition, there was no correlation between splenic size and the control of the eruption by a gluten-free diet. These findings are similar to the previous experience of splenic atrophy in coeliac disease in which no relation was found between the presence or absence of splenic atrophy and the clinical and laboratory findings in those patients (Marsh and Stewart, 1970).

Our results are yet further evidence that patients with dermatitis herpetiformis have coeliac disease, for not only is the small-intestinal lesion similar in both disorders but a similar abnormality of the spleen is present. The explanation of the splenic atrophy in dermatitis herpetiformis and coeliac disease is obscure at the present time but it may well be a further manifestation of an abnormality of the lymphoreticular system suggested by the finding of reticulin antibody in these disorders.

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Requests for reprints should be sent to Dr. J. E. Pettit.

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Lymphocytic Infiltration of Epithelium in Diagnosis of Gluten-sensitive Enteropathy

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Summary

The macroscopic appearance of the mucosa of the small intestine and the lymphocytic infiltration of the epithelium were studied in 27 patients with dermatitis herpetiformis and in 11 control subjects. The mucosa was abnormal in appearance in 13 of the patients and normal in 14 patients and in all the controls. In 25 (93%) of the patients the intraepithelial lymphocyte count was significantly raised compared with the controls. The increased lymphocytic infiltration of the epithelium in the patients probably represented an underlying immunological reaction of the small intestine to gluten, since the infiltration lessened in five out of six patients after a year on a gluten-free diet and in all of four patients after three years on a gluten-free diet.

Increased lymphocytic infiltration of the epithelium of the small intestine seems a surer sign of gluten sensitivity than the macroscopic appearance of the mucosa, and a diagnosis of gluten-sensitive enteropathy may no longer be excluded when the mucosa appears normal. Further evidence of the significance of increased lymphocytic infiltration is that patients with normal-looking mucosa but with raised intraepithelial lymphocyte counts often had low serum folate levels.

Introduction

About two-thirds of patients with dermatitis herpetiformis have an enteropathy judged by macroscopic and histological features of the mucosa of the small intestine (Marks *et al.*, 1966; Fraser *et al.*, 1967; Fry *et al.*, 1967). The enteropathy is similar to that of coeliac disease (Fry *et al.*, 1968, 1969; Shuster *et al.*, 1968; Marks and Whittle, 1969), which is usually diagnosed on the appearance of the intestinal mucosa (Booth *et al.*, 1962), and there is now mounting evidence of immunological abnormalities in both coeliac disease and dermatitis herpetiformis (Hobbs and Hepner, 1966; Crabbe, 1967; Douglas *et al.*, 1969; Soltoft, 1970; Seah *et al.*, 1971a, 1971b). Immunoglobulin deposits on the reticulin fibres of the skin have been found in dermatitis herpetiformis (Seah *et al.*, 1972), and lymphocytic infiltration of the lamina propria (Rubin *et al.*, 1960; Shiner and Drury, 1962) and of the intestinal epithelium (Ferguson and Murray, 1971) have been reported in coeliac disease.

We report here a study of the lymphocytic infiltration of the epithelium of the small intestine in a group of patients with dermatitis herpetiformis to see whether in those cases in which the mucosa was macroscopically normal there was abnormal infiltration and thus evidence of an immunological abnormality. The patients' folate status, as a sensitive indicator of functional disturbance of the mucosa of the upper small intestine, was also assessed.

Patients and Methods

Twenty-seven patients were studied. Dermatitis herpetiformis was diagnosed on the clinical and histological features of the rash, its clearance with dapsone therapy, and relapse on withdrawal of the drug.

An initial biopsy on mucosa from the small intestine of each patient was performed. Six of the 27 patients were put on a gluten-free diet for one year, and gluten was then reintroduced into the diet of five of them for a period of three months. At the end of the three months four of the five returned to a gluten-free diet for another three years. Further biopsies on mucosa from the small intestine were performed in the six patients at the end of their year on a gluten-free diet, in the five patients at the end of their three-month period on a diet containing gluten, and in the four patients at the end of their three-year period on a gluten-free diet.

The mucosa of the small intestine of 11 subjects who did not have dermatitis herpetiformis or coeliac disease was also studied.

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One specimen was obtained from a volunteer physician, four were obtained during partial gastrectomy for gastric ulcer, five were obtained from patients with eczema, and one was obtained from a patient with psoriasis.

The specimens were taken with the Crosby capsule from the duodenojejunal flexure under radiographic control in all patients. The macroscopic appearance was assessed with a dissecting microscope and the specimen photographed for record. It was then fixed in formol-saline and embedded in wax. Sections 7 μm thick were cut and stained with haematoxylin and eosin.

The epithelial cells and intraepithelial lymphocytes were counted in parts of the specimens where the plane of section was vertical through the epithelium and the underlying basement membrane. In villous specimens counts were made on the sides and tips of the villi, and in convoluted and flat specimens the surface cells were counted. (Casual observation indicated that glandular epithelium was much less heavily infiltrated with lymphocytes than that on the sides of villi or on the surface of flat and convoluted specimens.) The number of lymphocytes among 1,000 cells were counted in each sepcimen and the results expressed as lymphocytes per 1,000 epithelial cells. Note was taken of whether the lymphocytes were in a basal position, adjacent to the basement membrane, or at a higher level among the epithelial cells. Lymphocyte nuclei were usually easily distinguished from those of epithelial cells by their smaller size, more rounded overall shape, and deeper staining. Nuclei or nuclear fragments that could not be positively identified were ignored, as were occasional intraepithelial eosinophils.

Serum folate levels were determined by microbiological assay with *Lactobacillus casei* as the test organism (Waters and Mollin, 1961). The normal range is 6·0-21·0 ng/ml. Levels less than 3·0 ng/ml are regarded as abnormal and those between 3·0 and 5·9 ng/ml as borderline.

Red cell folate levels were estimated according to the modification of the serum folate assay described by Hoffbrand *et al.* (1966). The normal range is 160-640 ng/ml packed red cells.

Results

MACROSCOPIC APPEARANCES

The macroscopic appearance of the mucosa of the small intestine was flat in six of the 27 patients with dermatitis herpetiformis, convoluted in seven, leaves only in seven, and fingers and leaves in seven (Fig. 1). The macroscopic appearance in the 11 control subjects was leaves only in two and fingers and leaves in nine.

The initial macroscopic appearance of the mucosa in the six patients who had serial biopsies was flat in one, convoluted in two, leaves only in two, and fingers and leaves in one (see Table). After one year on a gluten-free diet the mucosa in the three patients in whom it had been abnormal (flat or convoluted) became normal (leaves only). After the reintroduction of gluten to the diet the mucosa became abnormal again in two of these three patients. The mucosa in the three patients in whom it was initially normal remained normal in two after one year on a gluten-free diet and on subsequent reintroduction of gluten, but in one patient (in whom the initial appearance was leaves only) the mucosa was convoluted after a year on gluten-free diet and remained so after the reintroduction of gluten.

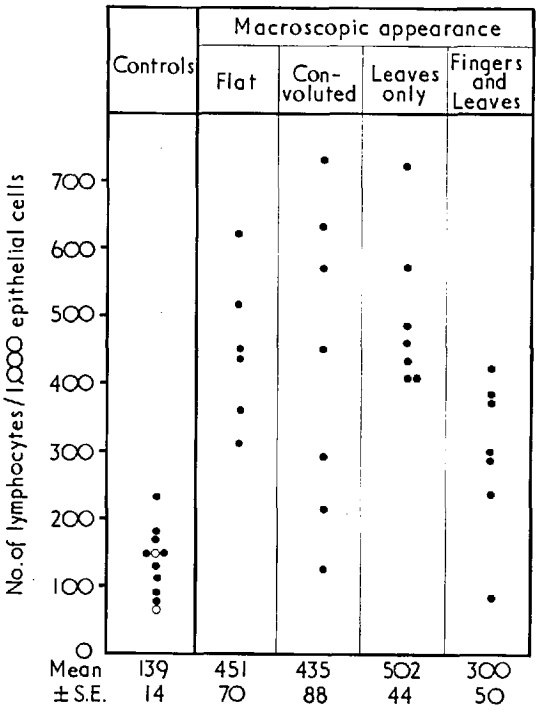

FIG. 1—Macroscopic appearance of small-intestinal mucosa in relation to lymphocyte counts in control and dermatitis herpetiformis specimens. O = Macroscopic appearance of leaves only in control group.

The mucosa of two of the four patients who were studied at the end of three years on a gluten-free diet had initially been abnormal and one of these two reverted to normal. There was no change in the two patients whose mucosa had appeared normal before their three-year period on gluten-free diet.

LYMPHOCYTE COUNTS

The mean lymphocyte count per 1,000 epithelial cells (Fig. 1) was 451 ± S.E. 70 (range 313 to 627) in the six flat specimens (Fig. 2), 435 ± S.E. 88 (range 130 to 739) in the seven convoluted specimens, 502 ± S.E. 44 (range 408 to 727) in the leaves only

Macroscopic Appearance of Mucosa of Small Intestine and Lymphocytic Infiltration of Epithelium in Relation to Gluten Withdrawal and Normal Diet

Case No.	Appearance of Mucosa				Lymphocyte Count per 1,000 Epithelial Cells			
	A	B	C	D	A	B	C	D
1	Con.	L.	Flat	Con.	295	308	238	221
2	Con.	L.	Con.	L.	635	375	369	277
3	F.L.	L.	F.L.	F.L.	374	304	307	293
4	L.	L.	L.	F.L.	408	209	438	103
5	L.	Con.	Con.	—	434	353	355	—
6	Flat	L.	—	—	399	348	—	—

Con. = Convoluted. L. = Leaves only. F.L. = Fingers and leaves.
A = Initial biopsy. B = After one year on gluten-free diet. C = Three months after reintroduction of gluten. D = After three years on gluten-free diet.

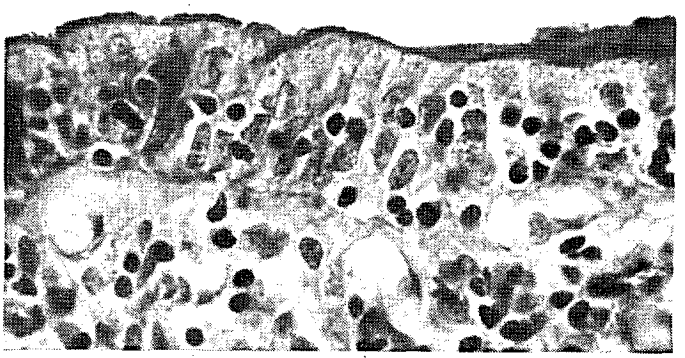

FIG. 2—Increased lymphocytic infiltration of epithelium in dermatitis herpetiformis. (Periodic-acid Schiff. × 400.)

specimens, and 300 ± S.E. 50 (range 89 to 422) in the fingers and leaves specimens.

In the 11 control specimens showing leaves only and fingers and leaves (Fig. 3) the mean count (Fig. 1) was 139 ± S.E. 14 (range 65 to 235).

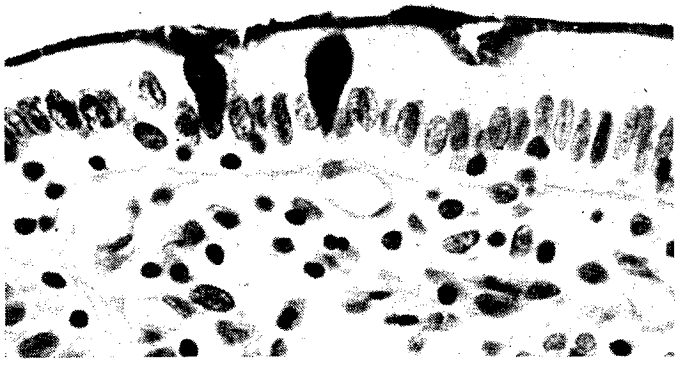

FIG. 3—Minimal lymphocytic infiltration of epithelium from volunteer physician. (Periodic-acid Schiff. × 400.)

The counts for each of the four groups of patients were widely scattered but in the control group they were very similar. The mean counts for the flat, convoluted, leaves only, and fingers and leaves specimens were not significantly different. Though the counts for the fingers and leaves specimens tended to be lower than for the other groups the difference was not significant ($P > 0.10$). The mean count for the fingers and leaves specimens, however, was significantly greater in the patients than in the control group ($P < 0.001$). Counts of less than 200 were found in only two specimens from patients, one of which had a convoluted and one a fingers and leaves macroscopic appearance. In only one of the 11 control specimens was the count above 200.

Serial Lymphocyte Counts in Six Patients.—The lymphocyte count had fallen in five and risen in one of the six patients after one year on a gluten-free diet (see Table). Nevertheless, in none did the count fall to below 200. The lymphocyte count rose in three and fell in two of the five patients in whom gluten was reintroduced and biopsy repeated three months later. There was a significant rise only in Case 4.

All four patients who were studied after three years on a gluten-free diet showed a fall in the lymphocyte count compared with their initial count. It was greatest in Case 4—from 408 to 103 (see Table).

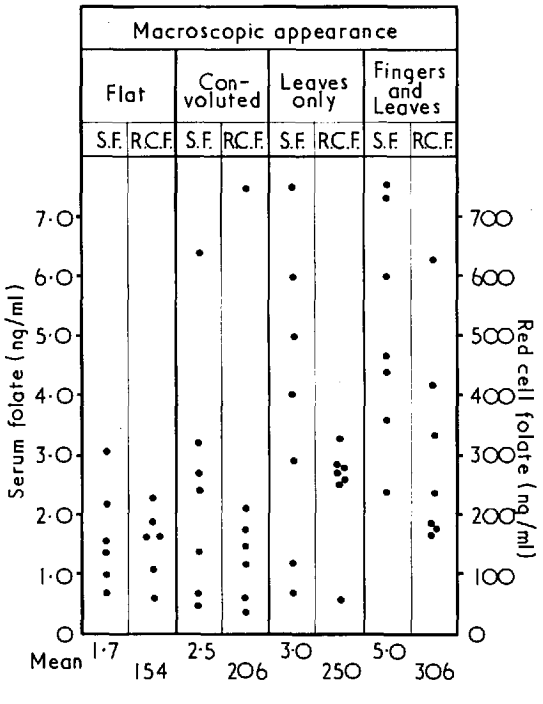

FIG. 4—Serum and red cell folate levels in relation to macroscopic appearance of mucosa. S.F. = Serum folate. R.C.F. = Red cell folate.

SERUM AND RED CELL FOLATE LEVELS

Some serum folate levels of less than 3·0 ng ml were found in all four groups of patients, but more often in patients with abnormal mucosa (Fig. 4). All the six patients with flat mucosa had low serum folate, and in five of them it was below 3·0 ng/ml. The red cell folate level was also subnormal in two of the six patients. The serum folate level was subnormal in six of the seven patients with a convoluted mucosa, and in five of them it was below 3 ng ml. Three of the seven also had subnormal red cell folate levels. Five of the seven patients with a leaves only appearance of the mucosa had low serum levels, and in three of them it was below 3·0 ng/ml, but the red cell level was subnormal in only one. Four of the seven patients with fingers and leaves appearance had a low serum level but it was below 3·0 ng ml in only one. All this group had normal red cell folate levels.

Discussion

There was a significant increase in the number of lymphocytes in the epithelium of the small intestine in 25 (93%) of the 27 patients with dermatitis herpetiformis. That the lymphocyte count was raised in 13 of the 14 macroscopically normal biopsy specimens is particularly interesting. Indeed, there was no significant difference in the degree of lymphocytic infiltration between specimens with a flat, convoluted, leaves only, or fingers and leaves appearance. In 61% of the 83 patients with dermatitis herpetiformis studied by four different groups of workers (Fraser *et al.*, 1967; Fry *et al.*, 1967; Marks *et al.*, 1968; Shuster *et al.*, 1968) the appearance of the mucosa of the small intestine was flat or convoluted. However, since the finding of circulating antireticulin antibody in patients with dermatitis herpetiformis (Seah *et al.*, 1971a, 1971b) Seah *et al.* (1972) have shown immunoglobulin deposits on the reticulin fibres of the upper dermis in all of 18 patients with dermatitis herpetiformis. This suggests that an immunological disorder is present in the skin in all patients with dermatitis herpetiformis, and if that is so some abnormality of the small intestine may also be present.

The report (Ferguson and Murray, 1971) that in untreated coeliac disease but not in other gastrointestinal diseases the lymphocytic infiltration of the small-intestinal epithelium is increased is probably evidence of an immunological abnormality at this site. Our finding of increased lymphocytic infiltration in 93% of the present patients strongly suggests that all patients with dermatitis herpetiformis have an immunological abnormality in the epithelium of the small intestine.

It seems that lymphocytic infiltration of the epithelium is a more sensitive marker than macroscopic appearance for detecting gluten sensitivity, and our study provides good evidence that lymphocytic infiltration is an indication of gluten sensitivity of the small intestine. Firstly, the skin condition of patients with dermatitis herpetiformis in whom the mucosa of the small intestine has the appearance of fingers and leaves or leaves only improves on a gluten-free diet and the serum folate level rises. When gluten is reintroduced the rash returns, larger doses of dapsone are needed, and the serum folate level falls (Fry *et al.*, 1969). Secondly, the present study shows that the lymphocytic infiltration lessens (irrespective of the original macroscopic appearance) when the patient takes a gluten-free diet, particularly

for a long period. Ferguson and Murray (1971) also reported that lymphocytic infiltration was less in patients with coeliac disease taking a gluten-free diet than in untreated patients.

Our findings together with those of Ferguson and Murray (1971) therefore raise the question of the criteria for diagnosing coeliac disease. Four out of seven of the present patients with a finger and leaf appearance of the mucosa and five of the seven with a leaves only appearance had subnormal serum folate levels. If these patients had not had a rash and had been found incidentally to have low folate levels they might have been labelled nutritional folate deficiency or temperate sprue (Cooke *et al.*, 1963). We suggest that some patients with folate deficiency and a normal macroscopic appearance of the small intestine may have a mild form of coeliac disease.

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