https://theses.gla.ac.uk/

Theses Digitisation:

https://www.gla.ac.uk/myglasgow/research/enlighten/theses/digitisation/

This is a digitised version of the original print thesis.

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Enlighten: Theses
https://theses.gla.ac.uk/
research-enlighten@glasgow.ac.uk

IMMUNOLOGICAL STUDIES OF BULLOUS PEMPHIGOID IN RELATION TO OTHER BULLOUS DISEASES

BY

Khalid Saleh Alkarawi, M.B., B.S., M.Sc.(Dermatology)

A thesis submitted in fulfilment of the
requirement for the degree of
Doctor of Philosophy in the
Department of Dermatology,
University of Glasgow

April, 1992

ProQuest Number: 10992064

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

ProQuest 10992064

Published by ProQuest LLC (2018). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Thesis
9248
copy 1

GLASGOW
UNIVERSITY
LIBRARY

CONTENTS

		Page
LIST OF TABLES		V
LIST OF FIGURES		VII
ACKNOWLEDGMENT		IX
SUMMARY		X
1	INTRODUCTION	
1.1	General Introduction	1
1.1.1	Clinical features	2
1.1.2	Clinical variants	5
1.1.3	Differential diagnosis	8
1.1.4	Pathology	11
1.1.5	Immunologic assessment	12
1.2	Immunofluorescence features of bullous pemphigoid and pemphigus	16
1.2.1	Bullous pemphigoid	17
1.2.2	Pemphigus	23
1.3	Immunoglobulin G subclasses	27
1.3.1	Physiochemical features	28
1.3.2	Biologic properties	30
1.3.3	Antibodies	33
1.3.4	Serum level abnormalities	34
1.4	Immunological characteristics of bullous pemphigoid and epidermolysis bullosa acquisita antigens	37
1.4.1	The basement membrane zone	39
1.4.2	Bullous pemphigoid	40
1.4.3	Epidermolysis bullosa acquisita	45
1.5	Dermatitis herpetiformis	49
1.5.1	Clinical features	49
1.5.2	Histopathology	51
1.5.3	Immunopathology	52
2	MATERIALS AND METHODS	

		Page
2.1	The demonstration of bullous pemphigoid and pemphigus antibodies by indirect immunofluorescence	56
2.1.1	The expression of bullous pemphigoid and pemphigus vulgaris antigens in normal skin	56
2.1.2	Comparison of different substrates for the titration of bullous pemphigoid and pemphigus antibodies	59
2.2	Comparison of IgG subclasses and complement binding activity in patients with bullous pemphigoid and pemphigus	61
2.2.1	Direct immunofluorescence studies	62
2.2.2	Indirect immunofluorescence studies	63
2.2.3	Indirect complement immunofluorescence	64
2.3	Immunofluorescence - split skin and Western immunoblotting studies	65
2.3.1	Indirect immunofluorescence using sodium chloride split skin	65
2.3.2	Direct immunofluorescence of NaCl-separated skin biopsy specimens	68
2.3.3	Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting studies	69
2.4	The specificity and sensitivity of endomysial antibodies	77
3	RESULTS	
3.1	The reactivity of bullous pemphigoid and pemphigus antibodies on different substrates	79
3.1.1	Variations in the distribution of bullous pemphigoid and pemphigus antigens in normal skin	79
3.1.2	The sensitivity of normal skin, primate oesophagus and guinea pig lip for the determination of anti-epithelial antibodies	84
3.2	The distribution of circulating and tissue bound IgG subclasses in pemphigoid and pemphigus	89
3.2.1	IgG subclasses in bullous pemphigoid	89
3.2.2	IgG subclasses in pemphigus vulgaris	93

		Page
3.3	Antigen specificities of anti-basement membrane zone antibodies	99
3.3.1	Localization of type IV collagen and type VII collagen on intact and NaCl-separated skin	99
3.3.2	Indirect immunofluorescence of bullous pemphigoid and epidermolysis bullosa acquisita antibodies on split skin	100
3.3.3	Immunofluorescence studies of NaCl-separated biopsy specimens	102
3.3.4	SDS-PAGE and Western immunoblotting	104
3.4	Endomysial antibodies in bullous diseases	106
4	DISCUSSION	
4.1	Selection of epithelial tissue for indirect immunofluorescence staining of bullous pemphigoid and pemphigus antibodies	110
4.2	Restricted distribution of IgG subclasses in bullous pemphigoid and pemphigus vulgaris	128
4.3	Immunofluorescence and Western immunoblotting studies of anti-basement membrane zone antibodies	150
4.4	IgA anti-endomysial antibodies in dermatitis herpetiformis	168
5	CONCLUSIONS	180
	REFERENCES	184

LIST OF TABLES

SECTION		
2.3.2	Table 1	Composition of running and stacking gel
2.3.2	Table 2	Composition of sample buffer
3.1.1	Table 3	Bullous pemphigoid antigen expression in normal skin
3.1.1	Table 4	Pemphigus vulgaris antigen expression in normal skin
3.1.2	Table 5	The reactivity of bullous pemphigoid sera on different substrates
3.1.2	Table 6	The reactivity of pemphigus vulgaris sera on different substrates
3.2.1	Table 7	IgG subclasses of tissue bound antibodies in bullous pemphigoid
3.2.1	Table 8	IgG subclasses of circulating antibodies in bullous pemphigoid
3.2.1	Table 9	Complement fixing antibodies in bullous pemphigoid
3.2.2	Table 10	IgG subclasses of tissue bound antibodies in pemphigus
3.2.2	Table 11	IgG subclasses of circulating antibodies in pemphigus
3.2.2	Table 12	Complement fixing antibodies in pemphigus
3.3.2	Table 13	The titration of pemphigoid and epidermolysis bullosa acquisita antibodies on intact and split skin
3.3.3	Table 14	Direct immunofluorescence of separated pemphigoid biopsies
3.3.4	Table 15	Bullous pemphigoid antigens recognized by Western immunoblotting
3.4	Table 16	The frequency of endomysial antibodies in bullous diseases
3.4	Table 17	Endomysial antibody titres in patients with dermatitis herpetiformis

4.1	Table 18	Body sites with the greatest expression of pemphigoid and pemphigus antigens
4.1	Table 19	Substrates used for the detection of bullous pemphigoid antibodies
4.1	Table 20	Substrates used for the detection of pemphigus antibodies
4.2	Table 21	The distribution of IgG subclasses in bullous pemphigoid
4.2	Table 22	The distribution of IgG subclasses in pemphigus vulgaris
4.4	Table 23	The incidence of anti-endomysial antibodies

LIST OF FIGURES

SECTION		
1.1.1	Figure 1	Bullous pemphigoid
1.1.1	Figure 2	Pemphigus vulgaris
1.4.1	Figure 3	The basement membrane zone
1.5.1	Figure 4	Dermatitis herpetiformis
3.1.1	Figure 5	The geometric mean of pemphigoid antibody titres on different skin specimens
3.1.1	Figure 6	The geometric mean of pemphigus antibody titres on different skin specimens
3.1.2	Figure 7	The reactivity of pemphigoid sera on different substrates
3.1.2	Figure 8 A - C	The staining pattern of pemphigoid antibodies on various substrates
3.1.2	Figure 9	The reactivity of pemphigus sera on different substrates
3.1.2	Figure 10 A - C	The fluorescence pattern of pemphigus antibodies on various substrates
3.2.1	Figure 11 A - C	Tissue bound IgG subclasses in bullous pemphigoid
3.2.1	Figure 12 A and B	Circulating IgG subclasses in bullous pemphigoid
3.2.1	Figure 13	Complement immunofluorescent staining with bullous pemphigoid serum
3.2.1	Figure 14 A and B	Tissue bound IgG subclasses in pemphigus vulgaris
3.2.1	Figure 15 A and B	Circulating IgG subclasses in pemphigus vulgaris
3.2.1	Figure 16	Complement immunofluorescent staining with pemphigus vulgaris serum
3.3.1	Figure 17 A and B	Indirect immunofluorescent staining using type IV and type VII collagen antibodies on intact normal skin

3.3.1 Figure 18 Staining pattern of type IV collagen antibody on sodium chloride separated skin

3.3.1 Figure 19 Staining pattern of type VII collagen antibody on sodium chloride separated skin

3.3.2 Figure 20 Immunofluorescent staining of bullous pemphigoid antibodies on separated skin

3.3.2 Figure 21 Immunofluorescent staining of epidermolysis bullosa acquisita serum

3.3.3 Figure 22 Sodium chloride separated bullous pemphigoid biopsy stained with type IV collagen antibody

3.3.3 Figure 23 Sodium chloride separated bullous pemphigoid biopsy stained with type VII collagen antibody

3.3.3 Figure 24 Direct IgG immunofluorescence of separated bullous pemphigoid skin specimen showing epidermal staining

3.3.3 Figure 25 Direct IgG immunofluorescence of separated bullous pemphigoid specimen showing epidermal-dermal staining

3.3.4 Figure 26 Nitrocellulose sheet stained with Ponceau S

3.3.4 Figure 27 Immunoblot of epidermal extract with bullous pemphigoid and control sera

3.4 Figure 28 Direct immunofluorescence of dermatitis herpetiformis

3.4 Figure 29 Dermatitis herpetiformis serum positive for endomysial antibodies

3.4 Figure 30 High magnification of monkey oesophagus section treated with endomysial antibody positive serum

3.4 Figure 31 Pemphigus serum negative for endomysial antibodies

3.4 Figure 32 Anti-smooth muscle antibodies of the IgA class

ACKNOWLEDGMENTS

I would like to express my deep gratitude to Professor R.M. MacKie for her supervision and guidance throughout the course of this work.

I am indebted to Dr. M.B. Hodgins and Dr. M. Seywright for their valuable help and advice.

My thanks are also expressed to the staff of the immunology laboratory, Department of Dermatology, for their kindness and support.

Finally, special thanks are due to my family for their understanding and encouragement during the course of this work and the preparation of this thesis.

SUMMARY

Bullous pemphigoid is a chronic autoimmune blistering disease of the elderly. The disorder is associated with linear deposition of IgG and complement along the epidermal basement membrane. Circulating IgG autoantibodies are present in the majority of patients and bind to a normal component of the basement membrane zone, the bullous pemphigoid antigen.

In the present study, certain immunological aspects of bullous pemphigoid were investigated and compared with pemphigus vulgaris and other immunologically mediated bullous diseases that may clinically resemble bullous pemphigoid, such as dermatitis herpetiformis and acquired epidermolysis bullosa.

The concentration of bullous pemphigoid and pemphigus vulgaris antigens in normal skin on different sites of the body was determined by indirect immunofluorescence using specimens from the face, trunk and limb and thirty serum samples. The end point titre of antibody reactivity in different areas was assessed. The greatest amount of bullous pemphigoid antigen was found in facial and limb skin whereas the pemphigus vulgaris antigen was more uniformly expressed over human skin from the various body sites.

Indirect immunofluorescence was also used to compare the sensitivity of normal human skin and two animal substrates, guinea pig lip and primate oesophagus, for the detection and titration of basement membrane zone and intercellular antibodies. All bullous pemphigoid and pemphigus sera produced a positive reaction on the three substrates but the highest antibody titres were observed when oesophagus sections were used in the immunofluorescence assays.

The distribution of tissue-bound and circulating IgG subclasses in patients with active pemphigoid and pemphigus was investigated by direct and indirect immunofluorescence using monoclonal antibodies specific for the four human IgG subclasses. In bullous pemphigoid IgG_4 was the most frequently detected subclass whereas IgG_3 was the rarest. Similarly, IgG_4 was the predominant antibody in the skin and serum from pemphigus vulgaris patients.

Indirect complement immunofluorescence was used to determine the complement fixing abilities of bullous pemphigoid and pemphigus antibodies. Complement fixing antibodies were observed in 60% and 41% in pemphigoid and pemphigus sera respectively. Analysis of the IgG subclasses in complement fixing bullous pemphigoid antibodies showed no compatibility with the complement activation characteristics of individual IgG subclasses whereas all complement fixing sera contained a complement activating IgG subclass.

The localisation of various basement membrane zone antigens including bullous pemphigoid, epidermolysis bullosa acquisita, type IV collagen and type VII collagen was studied using the sodium chloride split skin technique. Indirect immunofluorescence using cryostat sections of 1M sodium chloride-separated skin showed that type IV and type VII collagen which are associated with the lamina densa and sublamina densa stain the dermal edge of separated skin. Bullous pemphigoid antibodies produced an epidermal staining pattern whereas epidermolysis bullosa acquisita antibodies were associated with fluorescence of the dermal side of the split.

A distinct staining pattern of tissue-bound antibodies was also seen when bullous pemphigoid biopsies were separated by the same technique.

The bullous pemphigoid antigen was further characterised by performing sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western immunoblotting studies. The findings confirmed the presence of more than one bullous pemphigoid antigen and that the major antigen is the 220-240 KD protein.

Circulating IgA class anti-endomysial antibodies are directed against the connective tissue surrounding individual smooth muscle fibrils. The sensitivity and specificity of these antibodies for the diagnosis of dermatitis herpetiformis were investigated by determining their incidence in the sera of twenty dermatitis her-

petiformis patients and forty patients with other bullous disorders. The endomysial antibody test was positive in 73% of dermatitis herpetiformis patients on normal diet and in 20% of patients maintained on a gluten-free diet. The antibodies were not detected in any of the control subjects.

1. INTRODUCTION

1.1 GENERAL INTRODUCTION

The identification of bullous pemphigoid as a distinct subepidermal bullous disease was preceded by much confusion regarding its nosologic position in the classification of chronic bullous eruptions. Until its recognition as a well-defined entity, it had been confused with a variety of bullous diseases such as pemphigus, dermatitis herpetiformis and erythema multiforme. Bullous pemphigoid was first described as a separate disease by Lever in 1953[1]. The designation pemphigoid was given by Lever because the disease was clinically similar to pemphigus but with the histologic lack of acantholysis which is the essential feature of pemphigus. The term 'bullous' was added to distinguish it from another type of pemphigoid, benign mucosal pemphigoid (cicatricial pemphigoid).

Prior to Lever's classical description of bullous pemphigoid, it was grouped by some authors with pemphigus vulgaris and had various names including pemphigus vulgaris chronicus and pemphigus vulgaris benignus[2].

Bullous pemphigoid is now regarded as one of the pemphigoid spectrum of diseases which includes cicatricial pemphigoid, localized scarring pemphigoid and pemphigoid gestationis. The disease predominantly affects the elderly but occasionally occurs in young adults and children[2]. Unlike pemphigus vulgaris, there is no racial prevalence and males and females are equally affected[1,2].

1.1.1 Clinical Features

The characteristic clinical feature of bullous pemphigoid is the presence of large tense bullous lesions (Figure 1) which may measure several centimetres in size and have an irregular border. They usually contain a clear exudate but some blisters may be haemorrhagic. The tense bullae may remain intact for days. After rupture or reabsorption of blister fluid the bullous lesions become flaccid. The lesions do not rupture easily as in pemphigus (Figure 2). The blisters which do rupture leave denuded areas which tend not to increase in size as may occur in pemphigus vulgaris but show a tendency for healing without scarring[1,2]. The blisters of bullous pemphigoid may arise on normal appearing skin or on an erythematous base. The presence of clusters of bullae is not regularly seen but may be present in cases of extensive involvement. Although bullae predominate the clinical picture, patients may have a variety of other lesions such as erythematous patches which may have a serpiginous

Figure 1. Bullous pemphigoid. Tense blisters on normal-appearing and erythematous skin.

Figure 2. Pemphigus vulgaris. Trunk of a patient showing erosions and diffuse erythema.

configuration with central clearing. Irritation and cutaneous lesions suggestive of erythema multiforme or dermatitis herpetiformis may also precede the formation of bullous pemphigoid blisters[1,3]. The distribution of blisters in bullous pemphigoid is often generalized and may involve the entire skin surface; however, the most commonly affected areas are the lower abdomen, the inner aspects of the thighs, the groins, the axillae and the flexural surfaces of the arms and legs[1].

In contrast to pemphigus, the Nikolsky sign is negative in bullous pemphigoid[5]. The mucous membranes may occasionally be involved in bullous pemphigoid but mucosal lesions are rarely a presenting feature[1]. The incidence of mucous membrane involvement varies from 10% - 40% of cases[5-7]. The lesions are less severe than those of pemphigus vulgaris and are usually restricted to the mouth. When oral lesions are present they mostly affect the buccal mucosa and consist of small blisters that often remain intact and tend to heal rapidly if they rupture. Involvement of the vermilion border of the lips is rare. Other mucous membranes which may rarely be involved include conjunctivae, larynx, pharynx, oesophagus, vulva and anal mucosa[1]. The degree of pruritus in bullous pemphigoid is variable. It may be absent in some patients but in others it may be intense enough to require systemic treatment. Generalized pruritus may be a manifestation of bullous

pemphigoid. Cases have been reported with unexplained prolonged pruritus preceding the onset of classical bullous pemphigoid eruption[8,9].

There are many reports of bullous pemphigoid co-existing with autoimmune as well as with other disorders. In many of these reports the presence of two diseases in one patient may merely represent a coincidental association. However, the co-existence of bullous pemphigoid with diseases such as diabetes mellitus and psoriasis may be of clinical significance. Chaung et al.[10] studied the incidence of diabetes mellitus in patients with bullous pemphigoid in comparison with a selected control group of a similar age. The result of their retrospective assessment showed that 20% of bullous pemphigoid patients had diabetes mellitus prior to steroid therapy in contrast to only 2.5% of the control group.

The association of psoriasis and bullous pemphigoid has been suggested in several studies. In the majority of cases bullous pemphigoid was induced by psoralen plus ultraviolet A light (PUVA), ultraviolet B light and tar, and ultraviolet light and anthralin[11,12]. Others[13] have demonstrated a statistically significant increased incidence of psoriasis in bullous pemphigoid patients that was unconnected to therapy. In a retrospective case controlled study Grattan et al.[13] found psoriasis in 11% of 62 bullous pemphigoid patients but in none of the control group. The co-existence of bullous pemphigoid and

rheumatoid arthritis has been reported many times in the literature. One study suggested that the relationship between these two disorders represents more than a chance association[14]

Many cases of bullous pemphigoid have been described in the literature in which there was an association with malignancies of the lymphoreticular system, skin, lung, breast, genitalia, pancreas, kidney, gastrointestinal and urinary tracts[15]. However, Rook[16] has indicated that bullous pemphigoid and malignancy are diseases of elderly individuals and their coincidental association would not be surprising. Large series of bullous pemphigoid patients have been studied to determine the incidence of malignancy in bullous pemphigoid and to analyse the significance of this association. Most series comparing bullous pemphigoid with matched control groups showed no increased incidence of malignancy in bullous pemphigoid patients. Ahmed et al.[17] reviewed 33 cases of bullous pemphigoid and found only one patient (3%) to have an associated carcinoma. The incidence in an age-matched group was 4.2%. Stone and Schroeter[15] reviewed 73 patients with bullous pemphigoid and compared them with two age- and sex-matched control groups. Their results showed no increase in the incidence of malignancy in bullous pemphigoid than in the control groups. Some reports[5,7] have suggested that bullous pemphigoid patients with negative indirect immunofluorescence have a higher frequency of internal

malignancy in comparison with seropositive patients. However, controlled studies have not been performed to evaluate the significance of this association.

Bullous pemphigoid is a chronic but a much more benign disease than pemphigus vulgaris. The disease lasts a few months to several years and is characterized by periods of remission and exacerbation. Recurrences occurring after months or years of remission are usually less severe than the initial attack[1,5].

Bullous pemphigoid is a self-limiting disease and the patient's general health usually remains good. Unlike pemphigus vulgaris, the mortality rate is relatively low, even prior to the introduction of steroid therapy. However, the disease may be fatal in elderly debilitated patients[1,2]. Some authors have suggested that a prolonged clinical remission can be achieved in bullous pemphigoid patients treated with adequate doses of corticosteroids and/or immunosuppressive agents[18,19].

1.1.2 Clinical Variants

Localized bullous pemphigoid. It has been recognized for many years that bullous pemphigoid may start and remain a localized disease. This variant occurs in up to 30% of bullous pemphigoid patients[6,20,21]. In the series of Ahmed et al.[6], six of the seven patients reported had lesions confined to one extremity while in one patient the disease was localized to the scalp. Person et al.[21]

reported five patients with localized pemphigoid. The lesions were either restricted to one extremity or symmetrically involving two extremities. Localized bullous pemphigoid can be successfully treated with topical corticosteroids[22].

Childhood pemphigoid. Bullous pemphigoid rarely occurs in children. The clinical manifestations are similar to those of bullous pemphigoid in adults[23] . The disease must be distinguished from other bullous disorders of childhood including chronic bullous dermatosis of childhood and dermatitis herpetiformis. The diagnosis can only be confirmed by immunofluorescence studies.

Vesicular pemphigoid. In this variant the blisters are usually small, tense and grouped[24]. Although the clinical picture may be suggestive of dermatitis herpetiformis, direct immunofluorescence studies reveal IgG deposits along the basement membrane zone.

Polymorphic pemphigoid. Honeymann et al.[25] suggested this term for cases showing some clinical features of dermatitis herpetiformis without evidence of jejeunal atrophy. Direct immunofluorescence shows deposits of IgG or IgA at the basement membrane zone. Cases associated with IgG may represent a variant of bullous pemphigoid whereas those associated with IgA probably represent patients with linear IgA bullous dermatosis.

Vegetating pemphigoid is a rare variant of bullous pemphigoid. The disease is characterized by purulent vegetating lesions in the flexural areas which are similar to the lesions encountered in pemphigus vegetans. However, the direct immunofluorescent features are consistent with bullous pemphigoid[26].

Hyperkeratotic scarring pemphigoid is an unusual variant which is histologically characterized by subepidermal blister, hyperkeratosis, papillomatosis and acanthosis[22]. This variant closely resembles another type of bullous pemphigoid, pemphigoid nodularis, which is characterized by lesions which clinically and histologically mimic prurigo nodularis[27].

These variants have direct immunofluorescent features compatible with bullous pemphigoid.

1.1.3 Differential Diagnosis

Epidermolysis bullosa acquisita. This term was first introduced to describe patients with clinical manifestations of epidermolysis bullosa developing in adulthood with absent family history of epidermolysis bullosa[28]. Epidermolysis bullosa acquisita is clinically associated with trauma-induced blisters, skin fragility and erosions which mostly occur on the extensor surfaces and heal with milia and scars. Histologically, the disease is characterized by a subepidermal blister and a moderate inflammatory cell infiltrate composed primarily of neutrophils.

Routine immunofluorescent studies show features identical to those of bullous pemphigoid. An inflammatory variant of epidermolysis bullosa acquisita has been described[29]. It is associated with clinical features which may mimic bullous pemphigoid and thus result in diagnostic difficulties.

Dermatitis herpetiformis is a disease of young adults. It is characterized by intensely pruritic papules, vesicles or bullae. The lesions develop in a symmetrical distribution over the extensor surfaces, scalp and buttocks[30]. The histologic picture is characteristic but can occasionally be difficult to distinguish from bullous pemphigoid[31]. However, the two diseases can be differentiated by direct immunofluorescence which in dermatitis herpetiformis demonstrates granular IgA deposits in the dermal papillae. In addition, circulating anti-basement membrane zone antibodies cannot be detected in dermatitis herpetiformis[32]. Almost all patients have an associated gluten sensitive enteropathy and an increased prevalence of the HLA-B_8,DR_3 and DQ_{w2} HLA types[32,33].

Linear IgA bullous dermatosis represents a heterogeneous group of patients with a characteristic pattern of Ig deposition. Immunofluorescent studies in these patients demonstrate tissue-bound IgA in a linear fashion along the basement membrane zone, in contrast to the granular IgA pattern observed in dermatitis herpetiformis[32]. Linear IgA bullous dermatosis is also

characterized by a much lower incidence of jejeunal enteropathy and of HLA B_8 histocompatibility antigen[32]. Although linear IgA bullous dermatosis may present with clinical features suggestive of bullous pemphigoid, it affects a younger age group and shows tissue-bound IgA rather than IgG. Furthermore, circulating anti-basement membrane zone antibodies in linear IgA bullous dermatosis are of the IgA rather than the IgG class[32].

Cicatricial pemphigoid is a rare, chronic, subepidermal bullous disorder which mainly affects the mucous membranes. The oral and ocular mucosal surfaces are the most commonly involved sites. However, lesions may also occur in the larynx, oesophagus, genitalia and anal mucosa[5,34]. The major feature of cicatricial pemphigoid lesions is their tendency towards healing with scarring. Clinically, lesions appear as intact blisters or erosions. Involvement of the gingiva occurs commonly in the initial stages and may lead to the diagnosis of desquamative gingivitis[34]. The ocular mucous membranes are affected in approximately two-thirds of patients[2,5,34]. Involvement of the conjunctiva can result in ectropion, trichiasis, symblepharon formation and corneal ulcerations[2]. Cutaneous eruption occurs in 10%-30% of patients[2,34]. The lesions consist of tense blisters which are usually localized to the face and scalp. Occasionally, patients may have a generalized bullous eruption[35]. Localized scarring pemphigoid (Brunsting-Perry) is a variant of

cicatricial pemphigoid. It is characterized by a bullous eruption that remains limited to one area but in contrast to classical bullous pemphigoid it tends to heal with scarring[36].

1.1.4 Pathology

Light microscopy. The major histologic finding in bullous pemphigoid is a subepidermal blister[1]. Early blisters are found completely beneath the epidermis. However, in older lesions regeneration in the floor begins at the periphery and extends over the whole floor. This regeneration results in an apparent intraepidermal position of the blister[31]. The epidermis forming the roof of the blister is intact in early lesions, however necrosis may occur in older lesions[31]. The dermal changes are variable and depend on whether a biopsy was obtained from bullae arising on normal appearing or on erythematous, inflamed skin. It is recommended that biopsies be selected from an early blister which shows specific diagnostic features. Non-erythematous lesions show a sparse perivascular inflammatory infiltrate of monocytes and eosinophils not sufficient to establish a diagnosis[5]. Biopsies taken from erythematous lesions which may be urticarial are also unhelpful because the only pathological findings they show are dermal oedema and a perivascular accumulation of inflammatory cells that are rich in eosinophils. Conversely, inflammatory blisters show dense dermal in-

filtrate composed of numerous eosinophils with monocytes and neutrophils. The infiltrate is around the blood vessels, in the papillary dermis and in the blister cavity. As in dermatitis herpetiformis, the inflammatory infiltrate may extend to the tips of dermal papillae and form microabscesses. These consist predominantly of eosinophils rather than neutrophils[31].

Electron microscopy. Ultrastructural studies performed on specimens obtained from non-inflamed lesions show thinning of the basement membrane, disruption of anchoring filaments and blister formation within the lamina lucida which is localized between the plasma membrane of basal cells and the lamina densa[37]. The dermo-epidermal separation occurs as a result of the disruption and disappearance of anchoring filaments[37,38]. In inflamed bullous pemphigoid lesions the earliest ultrastructural finding consists of a marked dermal infiltrate of eosinophils and histiocytes. Subsequently, the basement membrane shows discontinuities with fragmentation and disappearance of anchoring fibrils, anchoring filaments and hemidesmosomes and degeneration of the basal cells[39].

1.1.5 Immunologic Assessment

Immunologic abnormalities of the skin and serum detected by the techniques of immunofluorescence proved to be of considerable value in the evaluation of acquired

bullous disorders. Prior to the use of immunofluorescence methods in the investigation of these disorders, immunologic studies were performed with agglutination tests[40]. However, the reactions obtained with these tests tended to be non specific. A major development in the understanding of the pathogenesis of blistering diseases occurred when it was demonstrated that bullous pemphigoid and pemphigus patients have tissue bound and circulating IgG antibodies[41,42]. Studies showed that serum antibodies are specific for the corresponding diseases and that they yield distinct responses demonstrable by the indirect immunofluorescence test. The characteristic reaction obtained with sera from bullous pemphigoid patients consists of staining the area of the basement membrane of stratified squamous epithelium whereas the antibodies in the sera of pemphigus patients react with the intercellular areas.

The existence of circulating anti-basement membrane zone antibodies in patients with bullous pemphigoid was first recognized by Beutner et al. in 1965[42]. Studies using the indirect immunofluorescence technique had revealed that sera of three patients, then diagnosed as having either bullous pemphigoid or dermatitis herpetiformis, contained antibodies directed against the basement membrane zone. In one patient who demonstrated positive basement membrane antibodies, direct immunofluorescent examination of a skin specimen showed in vivo binding of an-

tibodies along the basement membrane zone. The presence of bullous pemphigoid antibodies reactive with an antigen in the basement membrane was also reported by Chorzelski et al. in 1966[43]. These observations were of great significance in providing a more precise definition of the immunopathology of bullous pemphigoid and in confirming Lever's classification of bullous pemphigoid as a distinct bullous disorder. Further immunofluorescent studies of bullous pemphigoid extended the initial observations and confirmed the presence of anti-basement membrane zone antibodies in the majority of cases with active lesions[44,45]. In 1967 Jordon et al.[44] demonstrated that fourteen of sixteen bullous pemphigoid patients with active disease had circulating antibodies whereas the antibodies could not be detected in the sera of six patients who were in clinical remission. Thus, the presence of antibodies appeared to be related to the severity of the disease. The authors suggested that, like pemphigus vulgaris in which the titre of circulating intercellular antibodies decline or become undetectable in patients who are in remission, a similar correlation may exist in bullous pemphigoid.

In some patients the indirect immunofluorescence test may be negative in the initial stages whereas tissue bound antibodies are detectable by the direct technique. Jordon

et al.[44] suggested that initially the antibodies may be bound in vivo and thus are not available in detectable serum concentration.

Although most of the indirect immunofluorescence studies for the demonstration of anti-basement membrane zone antibodies were performed using primate skin and mucosa as a substrate, species specificity studies have shown that autoantibodies in the sera of bullous pemphigoid patients are not species specific and react, in addition to primate tissue, with human skin, rabbit and guinea pig epithelial tissue[46].

In addition to serum studies in bullous pemphigoid, Jordon et al.[44] investigated the presence of in vivo bound IgG using direct immunofluorescence. Positive staining was found within skin lesions in four of five patients and in three of them also within normal skin.

Studies by Chorzelski et al.[47] presented additional evidence confirming findings of tissue bound as well as circulating antibodies in the majority of bullous pemphigoid patients with active disease. Furthermore, sera obtained from typical cases of dermatitis herpetiformis were tested and showed no antibodies against the basement membrane zone, which underlined the specificity of these antibodies.

The immunological findings obtained by various investigators have focused attention on the possibility that bullous pemphigoid may be an immunologically mediated

disorder, and on the diagnostic and prognostic value of immunofluorescence studies in differentiating bullous pemphigoid from dermatitis herpetiformis and pemphigus.

1.2 THE IMMUNOFLUORESCENCE FEATURES OF BULLOUS PEMPHIGOID AND PEMPHIGUS

Considerable knowledge about the immunopathology of bullous pemphigoid and pemphigus has accumulated since the early studies of Beutner and Jordon[41] and Jordon et al.[42] which demonstrated both tissue bound and circulating autoantibodies in the skin and serum of patients with these conditions. Our understanding of the immunological mechanisms involved in these bullous disorders has increased substantially by studies using various techniques including direct and indirect immunofluorescence, complement indirect immunofluorescence, immunoelectron microscopy and immunochemical methods. These studies have been of utmost importance in establishing bullous pemphigoid and pemphigus as distinct immunologically mediated entities and in providing immunologic diagnostic criteria. This section will focus on the most widely used immunologic tests for the evaluation of bullous pemphigoid and pemphigus, the direct and indirect immunofluorescence assays.

1.2.1 Bullous Pemphigoid

Direct immunofluorescence. Since the discovery of immune deposits in bullous pemphigoid skin, direct immunofluorescence has been regarded as a basic and crucial method for the accurate diagnosis of this condition. Immunoreactants deposited in perilesional skin appear as a continuous fine linear pattern of fluorescence at the basement membrane zone. Granular or interrupted patterns may be seen in some areas. In biopsy specimens where there is epidermal/dermal separation the direct immunofluorescence test may be negative or the immune deposits may be present on the blister roof or on both sides of the blister[48]. The immunoreactants, IgG and the third component of complement C_3 can be detected in the majority of patients with bullous pemphigoid. In some cases IgG is absent and only C_3 is detectable[6]. The IgG deposits are present in up to 90% of cases and the linear deposits of C_3 can be detected in almost all patients with bullous pemphigoid [5,6]. Although C_3 is the most frequently encountered complement at the basement membrane zone, other components of the classical and alternate pathways have been observed including C_{1q}, C_4, C_5, properdin, and B_1H globulin[49,50]. IgA, IgM, IgD and IgE deposits have been reported[50]. Deposits of the immunoglobulins IgA and IgM are each detected in approximately 25% of patients[6,50]. IgD and IgE deposits have only been reported in association with IgG[6,48]. Ab-

sence of IgG may occur, especially in early disease, but IgG may be found a few weeks later or during recurrences[6]. Current evidence suggests that IgG and C_3 deposits disappear with remission[6]. The presence of IgA deposits in atypical cases of bullous pemphigoid may cause diagnostic difficulties, especially if IgG and IgA produce staining reactions which are of comparable intensity. The problem in such cases arises from the difficulty in differentiating bullous pemphigoid from linear IgA bullous dermatosis. A positive indirect immunofluorescence test for IgG anti-basement membrane zone antibodies suggests the diagnosis of bullous pemphigoid. Because of the importance of direct immunofluorescence in the diagnosis of bullous pemphigoid, the site from which a biopsy is obtained must be carefully selected. Linear deposits of IgG and C_3 may be absent in clinically blistered areas or from skin too distant from lesions. Therefore, the skin biopsy specimen is best obtained from an area approximately 2cm from a lesion[48]. Skin adjacent to a fresh bulla is preferred because in older lesions the basement membrane may be completely destroyed, resulting in a negative immunofluorescence finding. It has been suggested that the optimal site for a biopsy may be perilesional skin from a flexural area on the basis of the results obtained by Goldberg et al.[4] who demonstrated regional variations in the expression of bullous pemphigoid antigen with the highest concentrations apparently being in the intertriginous areas.

In another study, Weigand[51] studied the effect of anatomic region on the direct immunofluorescence test for bullous pemphigoid and found that immunofluorescence biopsies from the lower extremities are frequently negative. This observation, therefore, suggests that, whenever possible, the lower extremities are best avoided as a biopsy site for immunofluorescence studies in bullous pemphigoid. Although linear deposits of IgG and C_3, revealed by direct immunofluorescence, are considered characteristic of bullous pemphigoid, they have also been regularly demonstrated in a similar immunofluorescent pattern in other blistering eruptions including cicatricial pemphigoid, herpes gestationis, epidermolysis bullosa acquisita and bullous systemic lupus erythematosus[5,52-54]. Available techniques cannot reliably differentiate the deposits in bullous pemphigoid from those in cicatricial pemphigoid or herpes gestationis. However, differentiation from those in epidermolysis bullosa acquisita and bullous systemic lupus erythematosus is possible by direct immunoelectron microscopy[55,56].

Indirect immunofluorescence. This serologic technique is used in bullous pemphigoid for detecting anti-basement membrane zone antibodies in patients' sera. In this technique normal tissue is used as a substrate. Frozen tissue sections are overlaid with appropriate dilutions of serum. Fluorescein-labelled antihuman IgG is subsequently overlaid on the sections which labels the IgG

that previously bound to the basement membrane zone. The diagnostic value of immunofluorescence has been confirmed in many studies. They demonstrated that circulating anti-basement membrane zone antibodies can be detected in approximately 70% of patients with bullous pemphigoid. The incidence of a positive indirect immunofluorescence examination was 72% in Tuffanelli's study[57] of 81 patients and 67% among the 84 patients of Person and Rogers[5]. The anti-basement membrane zone antibodies were detected in the sera of 69% of 36 patients studied by Ahmad et al.[6] and in 72% of 124 cases reviewed by Hodge et al.[7]. Although a proportion of bullous pemphigoid patients, particularly at the early stages of the disease, have no demonstrable serum antibodies, follow-up examination of these patients' sera often yields a positive indirect immunofluorescence test[6]. The failure to demonstrate serum basement membrane zone antibodies in such patients has been attributed to the presence of unoccupied binding sites in the basement membrane zone and, therefore, circulating antibodies become detectable only after the saturation of these binding sites by basement membrane zone antibodies[6]. The serum antibodies produce a linear pattern of fluorescence at the basement membrane zone which mimics the pattern observed on direct immunofluorescence. Several authors have reported the presence of circulating antibodies that were not detected by routine indirect immunofluorescence. Kumar et al.[58] suggested that

"occult" anti-basement membrane zone antibodies can be detected by standard indirect immunofluorescence following prolonged incubation of bullous pemphigoid serum with whole skin. Hermann[59] detected circulating anti-basement membrane zone antibodies in the sera of four bullous pemphigoid patients only after treating serum with urea to dissociate circulating immune complexes. IgG antibodies bound to antigen can generate many molecules of C_3. The complement indirect immunofluorescence technique makes use of this amplification principle to increase the sensitivity of the assay system. In some patients antibodies capable of binding complement can be demonstrated in sera that are negative by routine indirect immunofluorescence. Millns et al.[60] reported complement fixing antibodies in seven bullous pemphigoid sera that were negative by standard indirect immunofluorescence. It appears that in such cases the anti-basement membrane zone antibodies are present in serum in concentrations too low to be detected by classical indirect immunofluorescence but may be detected by the more sensitive complement indirect immunofluorescence.

The variability in the incidence of seropositive bullous pemphigoid patients and the failure to detect the circulating antibodies in some cases may also be ascribed to the type of epithelial substrate used in the indirect immunofluorescence technique. Some authors have suggested that the type of epithelial tissue used has a major impact

on the result of serum testing for bullous pemphigoid antibodies[61]. In some cases the use of inappropriate epithelial substrate may lead to a false negative indirect immunofluorescence test[62]. There has been considerable controversy regarding the optimal choice of substrate for use in the indirect immunofluorescence assay. Some authors advocated the use of normal human skin, whereas others suggested that primate tissue is the ideal substrate for detecting bullous pemphigoid antibodies[6,61].

A correlation between bullous pemphigoid circulating antibodies and disease activity was suggested in the early reports of Jordon et al.[44] and Peck et al.[63]. In a subsequent study, Katz et al.[64] found correlation between titre of circulating anti-basement membrane zone antibodies and the severity of disease in eight bullous pemphigoid patients. Although some authors suggested that there is no direct correlation between antibody titres in bullous pemphigoid and disease activity[5,47,65], studies of large series of patients demonstrated that a general correlation exists. Successful control of the disease was associated with low or undetectable serum antibodies and relapses followed by the reappearance of basement membrane zone antibodies[6,66]. Maize & Provost[66] found monitoring serum and tissue bound antibodies to be useful in the management of bullous pemphigoid patients.

1.2.2 Pemphigus

Pemphigus is one of the most serious acquired bullous disorders. The disease is characterized by flaccid bullae which rupture easily and leave denuded areas that tend to materially increase in size, causing discomfort and serious complications[2]. The fragility of skin in pemphigus is due to loss of adhesion between epidermal cells as a result of antibodies binding to the cells[67]. Clinically, pemphigus may be divided into two groups depending on the location of the blister within the epidermis. In pemphigus foliaceus and pemphigus erythematosus the lesions form in a superficial, often subcorneal, location whereas in pemphigus vulgaris and pemphigus vegetans the blisters arise in a predominantly suprabasal location[2]. Both groups are associated with tissue bound and circulating autoantibodies directed against intercellular antigens [67].

Direct immunofluorescence. The deposition of IgG antibodies in the skin of patients with pemphigus was first reported by Beutner et al. in 1965[42]. The deposits were observed within skin lesions of five patients and in four of the five patients also within normal skin. In addition to their value in understanding the nature of immunological mechanisms involved in pemphigus, IgG intercellular deposits constitute a sensitive and reliable diagnostic test since they can be demonstrated in almost all cases with active disease[67]. The demonstration of these an-

tibodies is particularly useful in cases of suspected pemphigus with only a few lesions and negative indirect immunofluorescence[68]. Complement C_3 is often deposited in the intercellular areas of lesional skin whereas in normal skin complement deposits are uncommon. Judd and Lever[69] found C_3 deposits in normal skin in only 50% of their patients. In addition to IgG, other immunoglobulins may be detected in some patients. IgA and IgM are present in up to 50% of cases in mucosa and perilesional skin[69]. Studies have shown that the direct immunofluorescence remains positive for a long time after the disease has regressed. Judd and Lever[69] found a positive direct immunofluorescence test in 58 of 63 patients with pemphigus vulgaris and pemphigus foliaceus. Eleven of the 58 were in remission for more than a year and four of them for over five years. Most of the patients with a negative direct immunofluorescence test were free of lesions for several years.

Indirect immunofluorescence. Circulating antibodies in the sera of patients with pemphigus were originally reported by Beutner and Jordon in 1964[41]. Using indirect immunofluorescence, they demonstrated that sera from these patients contain antibodies against an antigen in the intercellular areas of stratified squamous epithelium. In a subsequent report Beutner et al.[42] confirmed their earlier observations and showed that the antibodies were detected in nine out of 16 patients. Three cases showed doubtful

staining and four were negative. It is now well recognized that these antibodies are of the IgG class and can be detected in the majority of patients with active disease[70]. Thus, the detection of these antibodies is useful for confirming the diagnosis and differentiating pemphigus from other bullous disorders. However, it must be noted that circulating intercellular antibodies have been occasionally described in diseases other than pemphigus, including various autoimmune diseases such as systemic lupus erythematosus[71], myasthenia gravis[71] and bullous pemphigoid[5]. The antibodies are also frequently detected in patients with burns[72].

Some authors have questioned the reliability of indirect immunofluorescence as a diagnostic technique in pemphigus. In an extensive study Judd and Lever[69] performed direct and indirect immunofluorescence on 63 patients to determine the reliability of these tests as diagnostic procedures. Of the 63 cases, 92% showed a positive direct immunofluorescence test whereas circulating intercellular antibodies were demonstrated in less than half the sera tested. They also described four patients with moderate or extensive disease who showed no evidence of serum antibodies but had a positive direct immunofluorescence test. On the basis of these results the authors considered the demonstration of tissue bound antibodies a more sensitive diagnostic method in pemphigus. However, since the type of substrate used in the indirect

immunofluorescence assay has a major influence on the outcome of the test, some authors attributed the results obtained by Judd and Lever to the inappropriate choice of antigenic substrate[73]. In another investigation, a retrospective analysis of 106 cases of pemphigus showed that 69 cases (65%) were positive by both direct and indirect immunofluorescence. A significant finding was the detection of circulating intercellular antibodies in another 28 patients in whom immunofluorescence examination of biopsies was negative. Only 9 patients were seronegative but were positive by direct immunofluorescence for in vivo-bound immune deposits[74]. The results, therefore, emphasize the reliability of indirect immunofluorescence as a laboratory method for the diagnosis of pemphigus. In addition to its diagnostic value, intercellular antibody titre determination has also been suggested to have a prognostic significance because of the tendency of serum antibodies to parallel disease activity. This association between the patient's clinical condition and serum antibody titre was first suggested by Beutner et al.[42]. This was confirmed in a subsequent report by Chorzelski et al.[75]. Sams and Jordon[65] performed serial antibody titre determinations on three pemphigus patients and showed a direct correlation with disease activity. Weismann[76] studied 24 patients and confirmed that relapses were associated with elevation of antibody levels and that clinical improvement was followed by a decline in antibody

titre. O'Loughlin[77] investigated 17 patients over a six year period and found that successful control of the disease was accompanied by a fall of serum antibodies to undetectable levels. In such cases treatment was reduced and eventually discontinued. Conversely, some investigators have questioned the use of serial antibody titres to gauge treatment or as a prognostic indicator although they acknowledged the existence of a general correlation between titres of intercellular antibodies and disease severity[78,79]. Others have disputed the existence of any such correlation. Anderson et al.[80] noted high antibody titres in clinically free patients whereas some patients with extensive disease had low or undetectable antibody titres. Similarly, Judd and Lever[69] found a negative titre in 41% of patients with lesions while 45% of patients free of lesions showed a positive titre. They concluded that antibody titre determinations were not reliable for evaluating disease severity.

1.3 IMMUNOGLOBULIN G SUBCLASSES

The division of gammaglobulins into subclasses was first reported in 1960 by Drey[81]. His study showed that human IgG is composed of a mixture of three subclasses. Subsequent investigations by Grey and Kunkle[82] and Terry

and Fahey[83] using antisera to isolated myeloma proteins showed that there were four subclasses of IgG. This subdivision of gammaglobulins was based on differences in the structure of the heavy chain of each of the subclasses. Although the original reports defined the IgG subclasses on the basis of antigenic differences between myeloma proteins, they also demonstrated that the antisera to myeloma proteins also reacted with the same epitopes in normal sera. The development of specific monoclonal antibodies has led to the accurate identification and measurement of the four subclasses. Many investigators have studied the IgG subclasses extensively and showed that each is characterized by specific structural, biological and physiochemical features.

1.3.1 Physiochemical Features

Studies of the physiochemical properties of IgG subclasses showed distinctive differences between them. Structurally, the molecular weight of IgG, IgG_2 and IgG_4 is about 51 kilo daltons (KD), whereas the molecular weight of IgG_3 is larger by approximately 5 KD [84]. The larger molecular weight of IgG_3 is the result of an unusually large middle part of the heavy chain, hinge region, which covalently links the two G_3 chains to each other. Another unique feature of IgG_3 is the presence of five interheavy chain disulfide bonds. IgG_1 and IgG_4 have two bonds while the heavy chains in IgG_2 are joined by

four bonds[85]. In addition, there is a high incidence of IgG_3 in monoclonal cryoglobulins. Grey[86] detected antigammaglobulin activity in five of seven myeloma proteins in patients with cryoglobulins. Four of these proteins were of the IgG_3 subclass. These observations indicate that cryoprecipitation of G_3 subclass occurs in a higher frequency than anticipated because this subclass represents only 7% of the total IgG.

The peptic cleavage pattern of the IgG subclasses was investigated and showed differences in their susceptibility to enzymic degradation. It was demonstrated that IgG_1 and IgG_3 are the fractions sensitive to papain digestion, separating into Fab and Fc fragments. On the other hand, IgG_2 and IgG_4 are resistant to papain[87]. Differences in the sensitivity to pepsin have also been noted. IgG_1 and IgG_2 show resistance peptic degradation. Conversely, IgG_3 and IgG_4 are sensitive to pepsin digestion with formation of $F(ab)_2$ fragments[88]. These observations indicate that IgG_3 is the subclass particularly susceptible to enzymic degradation. The relative resistance of IgG_2 to enzymic digestion may be attributed to the presence of four interheavy chain disulfide bonds[85]. Although the complete amino acid sequence of the various IgG subclasses has not been determined, studies of the amino acid composition of the heavy chains of the four subclasses showed a high degree of homology[89].

1.3.2 Biologic Properties

The introduction of specific monoclonal antibodies against human IgG subclasses had made it possible to effectively study and define their biological characteristics. Studies to determine the metabolic properties of the various IgG subclasses have been based on investigations using myeloma proteins due to the lack of purified normal proteins. The properties of IgG_1 are similar to IgG_3, whereas many of the IgG_2 characteristics are shared by IgG_4. The different IgG subclasses demonstrate variable ability to fix complement. IgG_1 and IgG_3 are potent complement activators while IgG_2 binds less well to complement. IgG_4 is unique in being a non complement fixing antibody[90]. It has been shown that myeloma IgG proteins of all subclasses bind to the C_{1q} subunit of the first component of complement and the affinity decreases in the order $IgG_3>IgG_1>IgG_2>IgG_4$[91]. Hautanen et al.[92] demonstrated that the complement components C_{3c} and C_{3d} bind strongly to IgG_1 and IgG_3, whereas no significant binding could be detected to IgG_2 or IgG_4.

Morrell[93] investigated the turnover of IgG subclasses and reported that the average biologic halflife ($T_{1/2}$) of IgG_1, G_2 and G_4 was 21 days. Spiegelberg et al.[94] also reported a similar $T_{1/2}$ for these subclasses, however they found T1/2 to be 11-12 days. In both studies G_3 was shown to have a shorter T1/2: 7-8 days. The shorter half life of G_3 is associated with a higher fractional catabolic

rate than other subclasses[93]. The reactivity of protein A, a staphylococcal wall antigen, with the various IgG subclasses is confined to IgG_1, G_2 and G_4[89]. This selective binding of IgG subclasses has been used for the purpose of isolating IgG_3. Interestingly, IgG_3 also fails to react with rheumatoid factors. IgG_1 shows the strongest reaction whereas lesser activity is seen with IgG_2 and IgG_4[95]. Active transfer across the placenta is a unique feature of gammaglobulins and it has been demonstrated that all four subclasses are transferable through the placenta[89]. In common with other biological functions of human IgG subclasses, there appear to be distinctive differences between the four subclasses with regard to their ability to bind to different white blood cells. Simmons[96] studied the distribution of surface and cytoplasmic IgG subclasses in peripheral lymphocytes and found IgG_2 and IgG_4 to be the predominant surface immunoglobulins, while IgG_1 and IgG_3 were more frequently expressed in the cytoplasm.

It has been suggested that IgG_2 and IgG_4 are "antigen recognizing units" and that their deficiency may be associated with severe recurrent pyogenic infections [97]. Endresen[98] demonstrated IgG receptors on the surface of human platelets which were specific for IgG_1 and IgG_3. A study of thrombocytopenic purpura showed that in 33 of 38 cases anti-platelet maternal antibodies belong to IgG_1 and IgG_2 subclasses[99]. Similarly, it has been shown that

receptors for IgG on human monocytes[100] and neutrophils[101] interact effectively with IgG_1 and IgG_3. Thus, these particular subclasses of IgG can act as effective opsonizing antibodies due to the presence of receptors for their Fc portions on phagocytic cells and their ability to activate complement.

Antibodies which have the ability to bind to mast cells and basophils have been termed homocytotropic antibodies. A definite type of homocytotropic antibodies are IgE class antibodies which remain in the skin for long periods and have been recognized as the predominant immunoglobulin class mediating hypersensitivity reactions[89]. It has been postulated that the IgG_4 subclass also has homocytotropic properties and contributes to allergic reactions[102]. Vijay and Perelmutter[103] showed that IgG_4 does bind to human basophils and leads to the release of histamine on challenge with appropriate antigens. Therefore it may participate in allergic reactions as IgE does. However, IgG_4 sensitizes the skin for a shorter period of time compared to IgE, and it is characterized by weaker binding to mast cells and resistance to heat at 56°C[89]. Conversely, van Toorenenbergen and Aalberse[104] could not confirm the results of Vijay et al.[103] and concluded that IgG_4 was incapable of sensitizing basophil leucocytes in vitro.

Some have regarded IgG_4 as a marker of allergy since elevated levels of this subclass are consistently higher in allergic patients compared to non allergic individuals. Increased levels of IgG_4 are only rarely found in healthy non allergic subjects[97]. In contrast, some studies suggested that these antibodies contribute to the beneficial effects of immunotherapy. Devey et al.[105] demonstrated that IgG_4 was the major antibody in the serum of hyposensitized patients and proposed that it may play a role in inhibiting hypersensitivity. Van der Giessen et al.[106] showed that IgG antibodies found in allergic individuals in response to immunization with grass pollen extract were predominantly of the IgG_4 subclass. They suggested that they act as allergen neutralizing antibodies.

1.3.3. Antibodies

Although antibody activities may be found in all subclasses, certain IgG subclasses may be produced in response to specific antigens. Recent studies have shown a restricted IgG response in patients with acquired bullous disorders such as pemphigus[107], bullous pemphigoid[108] and herpes (pemphigoid) gestationis[109]. A response restricted to specific subclasses has also been observed in other autoimmune disorders. Eisenberg et al.[110] studied patients with systemic lupus erythematosus and reported predominant restriction of anti sm antibody to IgG_1. They also found antiribonucleoprotein antibodies to

be predominantly IgG_1. Schur et al.[111] reported that antinuclear antibodies were of the IgG_1 and IgG_3 subclasses. Studies have also shown that anti DNA antibodies in the serum of systemic lupus erythematosus patients are predominantly of the IgG_1 and IgG_3 subclasses [110,111]. These findings are of particular interest in view of the complement activating potential of IgG_1 and IgG_3 fractions with subsequent complement mediated inflammation.

Antibodies to polysaccharides are primarily IgG_2[112]. This subclass also dominates immune reactions involving carbohydrate antigens[113]. Specific antibodies to both tetanus and diphtheria toxoids were found in IgG_1 and IgG_2[113]. When screening for IgG subclass restriction of hepatitis B surface antigen antibodies, IgG_3 was the most frequently found subclass besides the dominating IgG_1[112]. Although it is evident from these studies that some antigens stimulate the production of specific IgG subclasses, the cause for a restricted response to one or two subclasses is not known.

1.3.4 Serum Level Abnormalities

The levels of IgG subclasses in normal human serum have mostly been determined using the radial immunodiffusion technique. IgG_1 is the major subclass; it comprises 64-70% of the total gammaglobulins. IgG_2 and IgG_3 constitute 23-38% and 4-7% respectively. IgG_4 forms only 3-4% of the total human IgG[114].

Disturbances of the serum levels of the IgG subclasses have been reported by various investigators and consist of elevation, depression or complete lack of one or more of the subclasses. Many of the cases reported were associated with different types of immunodeficiencies. However, serum concentration abnormalities have also been described in a variety of other disorders. The deficiency patterns of IgG subclasses vary and total IgG may be depressed or normal.

Oxelius et al.[115] studied a group of immunodeficient patients and detected low levels of one or more IgG subclasses. The authors observed that the deficiency of one subclass may be associated with elevated levels of other IgG subclasses which indicates a possible compensatory mechanism. In such cases total IgG is normal or even elevated. The deficiency of specific IgG subclasses has been associated with severe chronic infections particularly affecting the upper respiratory tract. Van Loghem[116] reported that IgG_1 was undetectable in a family with three children. Two of the children were in good health while the third suffered from a chronic purulent ear infection. Furthermore, the author reported a healthy individual who had a selective IgG_2 deficiency, thus supporting the suggestion that a deficiency of one subclass is compensated for by other subclasses. A hereditary lack of IgG_2 and IgG_4 has been associated with frequent infections, particularly caused by Haemophilus influenzae[117].

A high susceptibility of IgG_2-deficient patients to recurrent infections by polysaccharide encapsulated bacteria such as pneumococci and Haemophilus influenzae has been reported by others[118]. Normal individuals without recurrent infections and complete absence of IgG_3 were described by Lefranc et al.[119]. It has been speculated that the absence of clinical disease in IgG_3 deficiency could be the result of IgG_3 having similar biologic properties to IgG_1, which represents 70% of total IgG. On the other hand, complete lack or marked deficiency of IgG_4 was found to be incompatible with good health. In a study by Heiner et al.[97] severe recurrent pyogenic infections, mostly of the respiratory tract, have been observed in children and adults with selective IgG_4 deficiency. The authors also reported very low levels of IgG_4 in five of 35 patients with bronchiectasis and suggested a causal relationship. Rarely, low but detectable levels of IgG_4 have been observed in healthy subjects. Disturbances of serum IgG subclasses have been noted in various systemic diseases. IgG_2 and/or IgG_3 may be depressed in patients with juvenile diabetes mellitus[117]. Mothers of infants infected with group B streptococcus have been shown to have low levels of IgG_1 and IgG_3[117]. Some investigators have reported high levels of IgG_4 in atopic disorders[120,121]. These findings are of importance in view of the evidence suggesting that IgG anaphylactic antibodies are implicated in some forms of human immediate

type hypersensitivity[122]. IgG_1 and IgG_3 were markedly elevated in a group of 12 adults and 14 children with systemic lupus erythematosus while IgG_2 and IgG_4 were undetectable[117]. Serum IgG subclasses have also been studied in some blistering disorders of the skin. Increased levels of serum IgG_4 have been reported in patients with pemphigus vulgaris[107] and bullous pemphigoid[108] whereas total serum IgG subclasses were not raised above normal in patients with endemic pemphigus foliaceus[123].

1.4 IMMUNOLOGICAL CHARACTERISTICS OF BULLOUS PEMPHIGOID AND EPIDERMOLYSIS BULLOUS ACQUISITA ANTIGENS

Like bullous pemphigoid, epidermolysis bullosa acquisita is a chronic subepidermal blistering disease characterized by circulating and in vivo bound antibodies to the basement membrane zone. In both disorders anti-basement membrane zone antibodies produce a morphologically indistinguishable linear pattern of staining by conventional direct and indirect immunofluorescence methods. This immunofluorescence feature is also shared by another subepidermal bullous disease, cicatricial pemphigoid[18].

This limitation of routine immunofluorescence technique has contributed to diagnostic difficulties and confusion regarding the nosology of these disorders.

Ultrastructural immunocytochemistry has played an important role in classifying bullous disorders that may have identical immunofluorescence findings by demonstrating that immune deposits may be localized in a specific area of the basement membrane zone[55,124].

Other techniques that have become useful in classifying subepidermal bullous diseases are immunoprecipitation and immunoblotting. These methods require that patients' sera contain circulating antibodies in order to detect the relevant antigen. Using these methods, some of the biochemical characteristics of different antigens can be established. Furthermore, it can be determined whether circulating antibodies from one patient recognize the same antigen as antibodies from another patient.

Because of the limited use of these techniques, the differential diagnosis is usually made on the differences in the clinical and histologic features of the disease. However, the reliance on these features may lead to incorrect diagnoses because patients with epidermolysis bullosa acquisita may have clinical and histologic features that are similar to those associated with bullous pemphigoid[29].

1.4.1 The Basement Membrane Zone

Knowledge of the structure of the basement membrane of human skin is essential for understanding the ultrastructural location of antigens in the different bullous disorders and the use of the newly developed monoclonal antibodies in the investigation and diagnosis of these diseases.

The basement membrane zone is the area where the epidermis is connected to the dermis (Figure 3). Ultrastructural studies have shown that it is composed of four structures[125]. The first of these structures is the plasma membrane of the basal cells. From the plasma membrane of keratinocytes extend electron dense structures called hemidesmosomes from which tonofilaments radiate. The second structure of the basement membrane zone is located beneath the plasma membrane and is referred to as the lamina lucida. It is an electron lucent, homogeneous area approximately 20-40nm in width. Anchoring filaments are collagen fibrils which extend perpendicularly across the lamina lucida and insert into the lamina densa. Laminin is a high molecular weight non collagenous glycoprotein that has been localized to the lamina lucida[126]. Beneath the lamina lucida is the lamina densa which is the third major component of the basement membrane zone. In contrast to the lamina lucida, the lamina densa is wider (30-60nm) and has an electron dense amorphous appearance. Type IV collagen is a distinct an-

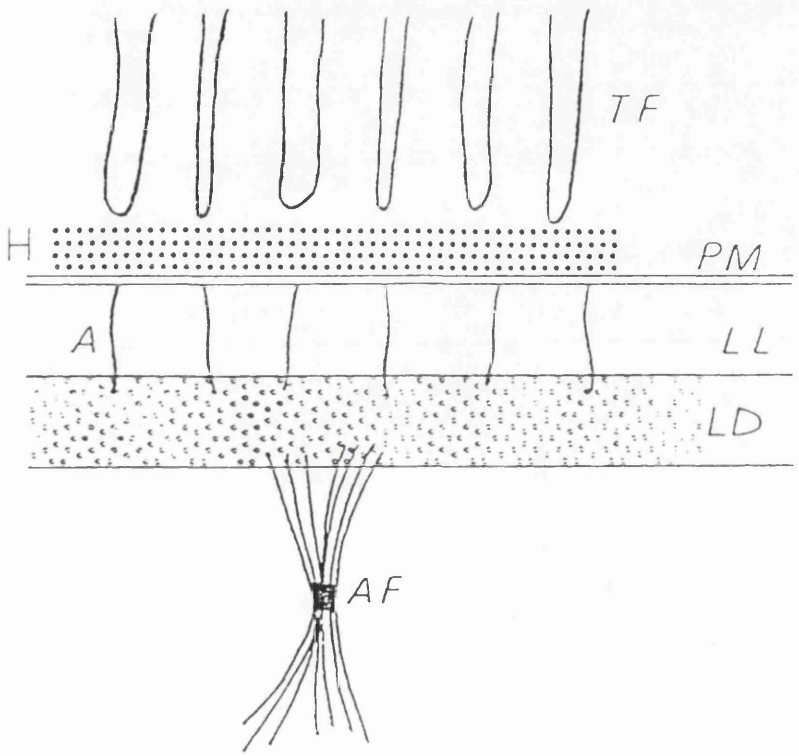


Figure 3. Schematic structure of the dermal-epidermal junction (adapted from reference 125). TF = tonofilaments, H = hemidesmosomes, PM = plasma membrane, A = anchoring filaments, LL = lamina lucida, LD = lamina densa, AF = anchoring fibrils.

tigenic component which is located within the lamina densa[127]. The fourth structure of the basement membrane zone is the sublamina densa area which is located just beneath the lamina densa and includes different types of fibrillar structures. Anchoring fibrils are electron dense structures that radiate from the lower aspect of the lamina densa and extend into the upper papillary dermis[125]. Recent studies have shown that the major structural component of anchoring fibrils is type VII collagen[128,129].

1.4.2 Bullous Pemphigoid

The bullous pemphigoid antigen is a normal constituent of the basement membrane zone. It is mostly detected in tissues containing stratified squamous epithelium but is also found in epithelial basement membranes of urethra, bladder, bronchi and gall bladder[46]. The antigen is defined immunologically by antibodies present in the sera of patients with bullous pemphigoid which bind to the basement membrane of normal stratified squamous epithelial substrates.

Studies have shown that epidermal cells in culture could synthesize the bullous pemphigoid antigen[130]. Stanley et al.[131] confirmed this finding and demonstrated that sera from different patients with bullous pemphigoid react with the same antigen.

The localization of bullous pemphigoid antigen has been studied by immunoelectron microscopy techniques. It was shown that in vivo bound immunoglobulins were mainly confined to the lamina lucida[55,132]. Similarly, indirect immunoelectron microscopy demonstrated that circulating bullous pemphigoid antibodies bind the lamina lucida[133]. However, more recent investigations suggested that bullous pemphigoid antigen is also associated with the hemidesmosomes[134,135]. When epidermal cells in suspension are air dried and treated with bullous pemphigoid serum and FITC conjugated antihuman IgG a polar rim of fluorescence is observed[136,137]. It has been proposed that this indicates that the bullous pemphigoid antigen is localized to the dermal pole of basal cells[134]. Subsequent studies suggested that the antigen is a molecule with an intracellular component associated with an extracellular portion localized within the lamina lucida. The actual localization of the antigen in the basement membrane zone and within normal keratinocytes has been determined by several investigators. In a study by Mutasim et al.[134] viable murine epidermal sheets, separated below the lamina densa using dithiothreitol, incubated with bullous pemphigoid serum, and sectioned and treated with antihuman IgG, did not show staining at the basement membrane zone. Conversely, typical linear fluorescence was seen when the sheets were processed by standard indirect immunofluorescence. The authors also studied the effects of per-

meabilization of cell membrane on the detection of antigen. The epidermal sheets were treated with a permeabilizing agent and then incubated with bullous pemphigoid serum prior to sectioning and processing. Linear staining was observed along the basement membrane zone.

Because the plasma membrane of viable cells is impermeable to antibody molecules, the results of these experiments were regarded as evidence that an intracellular bullous pemphigoid antigen exists in epidermal sheets. Similar results were obtained using basal cell suspensions. It was demonstrated that basal cells with permeable membranes bind bullous pemphigoid antibodies. Similar observations were reported by Regnier et al.[138]. Furthermore, they extracted the bullous pemphigoid antigen from non-viable basal cell populations that stain extracellularly with bullous pemphigoid serum and from another pool of non-permeabilized keratinocytes. When the two were compared by immunoblotting, it was shown that the bullous pemphigoid antigen from both extracts was 220kd. Immunoelectron microscopy studies using permeabilized epidermal sheets and bullous pemphigoid serum show immunoreactants almost exclusively intracellularly as clumps along the dermal pole of the basal cell plasma membrane. The sites of deposition corresponded to the location of hemidesmosomes. Only faint and focal staining was noted in the lamina lucida[134]. When trypsin dissociated per-

meabilized basal cells were examined by immunoelectron microscopy IgG deposits were observed on intracytoplasmic vacuoles which represent "internalized hemidesmosomes"[134].

The association of bullous pemphigoid antigen with the intracellular part of hemidesmosomes has been confirmed by others[138]. Other indirect immunoelectron microscopy studies using both peroxidase and colloidal gold techniques revealed that bullous pemphigoid antigen is localized inside the basal cell and preferentially associated with the hemidesmosomal plaques and tonofibrils [135].

The various studies which demonstrate an intracellular location for bullous pemphigoid antigen are in contrast with the earlier reports[132,133] which suggested that the antigen is localized within the lamina lucida. Mutasim et al.[139] have recently investigated the discrepancy between reported ultrastructural binding sites of bullous pemphigoid antibodies. Antibody binding was examined by placing viable skin and bullous pemphigoid serum in organ culture. Under these conditions direct immunoelectron microscopy showed immune deposits located exclusively in the upper lamina lucida, predominantly beneath the hemidesmosomes. Conversely, indirect immunoelectron microscopy using skin cryosections demonstrated intracellular binding of anti-basement membrane zone antibodies in association with the hemidesmosomes. The detection of an extracellular bullous pem-

phigoid antigen in the direct technique was attributed to the intact plasma membrane of viable cells; consequently, bullous pemphigoid antibodies had access only to the extracellular antigen. It was concluded that the bullous pemphigoid antigen is a transmembrane protein with bullous pemphigoid antibodies capable of binding both an extra- and an intracellular antigen.

Studies using immunochemical techniques have recently been used to determine the biochemical characteristics of bullous pemphigoid antigen. Initial studies by Diaz et al.[140] in 1977 demonstrated a 20kD protein extracted from normal human epidermis separated from the dermis by sodium thiocyanate. Using immunoprecipitation from buffer saline extracts of cultured epidermal cells, the bullous pemphigoid antigen was identified as a high molecular weight protein with disulfide chains of approximate molecular weight of 220kD. The same antigen was precipitated by sera from different bullous pemphigoid patients which suggested the existence of a single bullous pemphigoid antigen[131]. The same group also demonstrated that the bullous pemphigoid antigen is distinct from laminin and fibronectin which are also high molecular weight components of the basement membrane[131,141]. In a subsequent study Stanley et al.[142] showed that bullous pemphigoid antigen can be directly extracted from human skin. Using sodium dodecyl sulfate extracts of suction derived epidermis and Western immunoblotting, the bullous pem-

phigoid antigen was demonstrated to consist of two non-disulfide linked polypeptide chains with molecular weights of approximately 220-240kD.

1.4.3 Epidermolysis Bullosa Acquisita

In 1971 Roenigk et al.[28] reviewed the literature on epidermolysis bullosa acquisita and reported three cases. They suggested that this acquired blistering disease is a distinctive condition and established four criteria for the diagnosis which included (a) trauma-induced bullae over the joints of the hands, feet, elbows and knees, atrophic scars, milia and nail dystrophy; (b) adult onset of the disease; (c) negative family history of epidermolysis bullosa; (d) exclusion of other bullous diseases including porphyria cutanea tarda, pemphigus, bullous pemphigoid, dermatitis herpetiformis and bullous drug eruption. Histologically, epidermolysis bullosa acquisita is characterized by a subepidermal blister. Biopsies from areas of scarring show homogenization of the upper dermis with or without a mild mononuclear perivascular cell infiltrate[28]. Inflammatory lesions may be difficult to differentiate from bullous pemphigoid. They are associated with dermal oedema and a moderate to intense inflammatory infiltrate composed of neutrophils, eosinophils and monocytes. Occasionally, the infiltrate consists mainly of neutrophils[143]. Immunofluorescence studies

showed that patients with epidermolysis bullosa acquisita had immunofluorescence features that resemble those of bullous pemphigoid and cicatricial pemphigoid[53].

The clinical features of epidermolysis bullosa acquisita are markedly variable and may simulate those characteristic of other bullous disorders. This variation in clinical presentation and the similarity between routine histologic and immunofluorescence features in epidermolysis bullosa acquisita and other subepidermal blistering diseases led to the suggestion that epidermolysis bullosa acquisita was not a distinct entity and that its clinical features are manifestations of other specific diseases such as bullous pemphigoid and cicatricial pemphigoid [144,145]. However, subsequent immunoelectron microscopy studies demonstrated features different from those in bullous pemphigoid and cicatricial pemphigoid. Nieboer et al.[56] showed that immune deposits were found in the upper dermis beneath the lamina densa. This is in contrast to pemphigoid, where the immune reactants are deposited above the lamina dense[132,133]. Studies by Yaoita et al.[124] confirmed the immunoelectron microscopy findings in epidermolysis bullosa acquisita and showed that circulating anti-basement membrane zone antibodies in epidermolysis bullosa acquisita patients also bind to the sublamina densa area. The authors proposed a modification to the diagnostic criteria originally outlined by Roenigk et al.[28]: (a) mechano-bullous lesions

with scarring and milia; (b) negative family history of epidermolysis bullosa; (c) subepidermal blister histologically; (d) deposition of IgG at the basement membrane zone; (e) IgG deposits in the sublamina densa zone.

In addition to the immunoelectron microscopy differences between bullous pemphigoid and epidermolysis bullosa acquisita, immunoblotting and immunoprecipitation studies have shown that the two disorders are biochemically distinct. The antigen reactive with epidermolysis bullosa acquisita autoantibodies is a normal constituent of the skin basement membrane. This antigenic component has recently been partially characterized by Woodly et al.[146]. Crude basement membrane zone extracts prepared from normal human skin were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and reacted in a Western blot assay with epidermolysis bullosa acquisita sera. Antibodies in epidermolysis bullosa acquisita serum samples detected a 290kD and 145kD protein. Monoclonal antibodies that specifically bind to the epidermolysis bullosa acquisita antigen have been reported. The antibodies demonstrate an immunofluorescence and immunoelectron microscopy binding pattern which is identical to that produced by polyclonal antibodies. Moreover, these antibodies identify the 290kD and 145kD proteins in the basement membrane extracts of human skin[147-149].

Stanley et al.[150] showed that epidermolysis bullosa acquisita antigen is synthesized in culture by human keratinocytes and fibroblasts. They identified the 290kd chain of the antigen by immunoprecipitation studies using monoclonal and polyclonal epidermolysis bullosa acquisita sera. Further studies indicated that the target antigen for epidermolysis bullosa acquisita antibodies is the carboxyl terminal region of type VII collagen[147,151]. Using Western blotting, type VII procollagen and basement membrane extracts of skin, Woodly et al.[151] studied the labelling patterns produced by polyclonal sera from epidermolysis bullosa acquisita patients, the monoclonal antibodies to epidermolysis bullosa acquisita antigen, and monoclonal antibodies to the carboxyl terminal of type VII collagen. They showed that all antibodies detect an antigen at 290kD. Furthermore, all epidermolysis bullosa acquisita sera and monoclonal antibodies failed to react with pepsinized type VII collagen which consists of a triple helical molecule but lacks the carboxyl terminal domain..

1.5 DERMATITIS HERPETIFORMIS

Dermatitis herpetiformis was first described by Duhring in 1884[152]. Under this category he described a pruritic disorder with polymorphous eruption of erythematous papular, urticarial and vesicular lesion often in a characteristic herpetiform pattern. Duhring, however, included in his original publication not only what is now considered as dermatitis herpetiformis but also bullous pemphigoid which was termed bullous dermatitis herpetiformis. The confusion between dermatitis herpetiformis and bullous pemphigoid continued until they were separated by Lever in 1953. In addition to the clinical and histological[153] differences between the two diseases, it was demonstrated that sulfapyridine was effective in controlling dermatitis herpetiformis but had minimal or no effect on bullous pemphigoid[154]. The detection of granular immunoglobulin deposits in the skin of dermatitis herpetiformis patients was the first indication that the disease may be immunologically mediated[155].

1.5.1 Clinical Features[30]

Unlike bullous pemphigoid, dermatitis herpetiformis tends to predominantly affect males. Although the disease has been reported in various age groups, it mostly occurs in the second to fourth decades. The lesions of dermatitis herpetiformis usually develop in a symmetrical

fashion and regularly involve the extensor surfaces. The sites of predilection include the elbows, extensor forearms, knees, buttocks and scalp. The primary lesion of dermatitis herpetiformis is an erythematous papule or a papulovesicle (Figure 4). Large bullae are not usually present. Bullous lesions may occur in some patients after the withdrawal of long-term therapy[156]. The "herpetiform" grouped arrangement of lesions is highly characteristic of the disease. However, scattered, non-grouped lesions are often present. The Koebner phenomenon has been reported in dermatitis herpetiformis[157] with lesions occurring in areas of trauma or prolonged pressure. The demonstration of this phenomenon may account for the distribution of lesions on the scalp, elbows, knees and buttocks. The majority of dermatitis herpetiformis patients experience an intense degree of pruritus and burning. In many patients these symptoms precede the eruption of lesions which leads to excoriation and crusting. Occasionally, excoriated lesions are the only presenting manifestation, and the clinical diagnosis is reached on the basis of the characteristic distribution of lesions. Hyperpigmentation and hypopigmentation commonly occur following the involution of lesions[30,156].

It is now well recognized that the majority of dermatitis herpetiformis patients have some degree of gluten sensitivity which causes intestinal abnormalities of the coeliac disease type[158]. This strong association between

Figure 4. Dermatitis herpetiformis. Grouped papulo-vesicles involving the extensor forearm.

gluten ingestion and skin disease indicates that the intestinal defect may have an essential role in the pathogenesis of dermatitis herpetiformis. A gluten-free diet is associated with control of the rash and reversal of the pathological changes in the gut[159].

1.5.2 Histopathology

The histologic features of clinical non-vesicular lesions consist of accumulations of neutrophils and some eosinophils at the tips of dermal papillae[153]. With progressive infiltration of neutrophils in papillae microabscesses form. The development and progressive increase of microabscesses eventually leads to bulla formation. The cellular infiltrate of the underlying dermis is composed of neutrophils and some eosinophils. Many neutrophils show fragmentation of their nuclei, "nuclear dust"[31,160]. The dermal blood vessels are usually surrounded by lymphohistiocytic infiltrate as well as neutrophils and eosinophils[160].

The neutrophilic papillary microabscesses are diagnostic of dermatitis herpetiformis. A study of Kint et al.[161] showed that these lesions were observed in all 14 patients tested. However, in a study of 105 biopsy specimens by Connor et al.[160], the papillary microabscesses were present in only 52% whereas leukocytoclasia was noted in 77% of specimens. Lever[31] concluded that the biopsies were not obtained from clini-

cally non-vesicular areas which regularly show the neutrophil microabscesses. Occasionally, the differentiation between dermatitis herpetiformis and bullous pemphigoid may be difficult. Some lesions may show a subepidermal blister with a cellular infiltrate that contains a significant number of eosinophils, thus resembling the histologic picture of bullous pemphigoid[162]. Microabscesses may also be seen in bullous pemphigoid. They usually contain eosinophils, but at times may be composed largely of neutrophils[5].

1.5.3 Immunopathology

The presence of immunoreactants in the skin of patients with dermatitis herpetiformis was first reported by Cormane[155] in 1967, who found immunoglobulins in both involved and uninvolved skin. In a subsequent study, Van der Meer[163] reported immunoglobulins in the uninvolved skin in 10 of 12 patients. The immunoglobulin class was IgA. IgG was also detected in some patients. These findings confirmed that dermatitis herpetiformis is distinct from other blistering eruptions and that it may be immunologically mediated. The demonstration of IgA antibodies in the skin of suspected cases of dermatitis herpetiformis is regarded as an essential criterion for the diagnosis[164,165]. IgA deposits are found with or without other immunoreactants in more than 95% of cases when uninvolved skin is tested[164]. Different patterns of IgA

deposition are seen in the skin of dermatitis herpetiformis patients. Chorzelski et al.[166] were the first to describe the localization of IgA deposits at different sites. The majority of patients show IgA almost exclusively located in the dermal papillae. The appearance of deposits is usually granular. However, some patients may have a fibrillar component to the IgA deposits which appear as string of fluorescence. Infrequently, IgA deposits may be present in a homogeneous linear pattern along the basement membrane zone. Cases associated with this pattern were initially considered as variants of dermatitis herpetiformis[164]. However, linear IgA deposition is now considered a separate disease termed linear IgA bullous dermatosis[32]. A small proportion of patients show a granular linear pattern which appears as a fine granular line restricted to the basement membrane zone[32].

Occasionally, some biopsy specimens from suspected cases of dermatitis herpetiformis yield a negative direct immunofluorescence test for IgA. It has been suggested that patients who lack IgA deposits do not have dermatitis herpetiformis[167]. It must be noted, however, that the selection of the biopsy site in patients with dermatitis herpetiformis is important. Specimens of inflamed skin or a blister usually yield a false negative immunofluorescence result[166]. It must also be emphasized that in some patients repeated biopsies and studies of serial sections may be required before a positive result is obtained[167].

Zone et al.[168] suggested that uninvolved, distant skin tends to yield decreased amounts of IgA. Deposits of IgA could not be detected in such locations in four of 14 dermatitis herpetiformis patients who were in active disease, However, granular IgA deposits were observed in all cases in biopsies obtained from normal-appearing skin that was approximately 5mm from an active lesion[169]. Marks[158] questioned the reliability of direct immunofluorescence for IgA as a diagnostic test in dermatitis herpetiformis and indicated that there are patients who met all other diagnostic criteria, except IgA, and respond to sulfapyridine or dapsone therapy. Fry et al.[167] studied similar cases and found that an alternative diagnosis can be reached in the majority of cases.

Complement, most commonly the third component, is present in a distribution similar to IgA[163]. Immunoglobulins other than IgG are occasionally found in the skin in dermatitis herpetiformis. These are also seen in a distribution identical to IgA. In a study of 19 patients, Chorzelski et al.[166] found IgG in six patients and IgM in five patients. In another study of 78 patients, IgG and IgM were detected in two and seven patients respectively[164].

A variety of circulating antibodies have been identified in dermatitis herpetiformis. Gastric parietal cell antibodies, thyroid antibodies and antinuclear antibodies have been detected in variable frequencies[170]. Serum from

patients with dermatitis herpetiformis may also contain antibodies against reticulin and gliadin and the more recently described antiendomysial antibodies[170-172].

2. MATERIALS AND METHODS

2.1 THE DEMONSTRATION OF BULLOUS PEMPHIGOID AND PEMPHIGUS ANTIBODIES BY INDIRECT IMMUNOFLUORESCENCE

2.1.1 The Expression of Bullous Pemphigoid and Pemphigus Vulgaris Antigens in Normal Skin

The expression of bullous pemphigoid and pemphigus vulgaris antigens was assayed by indirect immunofluorescence using specimens of normal human skin from different body regions as a substrate.

Skin specimens. The specimens were obtained from the edges of surgically excised benign skin lesions. No skin was taken from patients with blistering or autoimmune disease. Antigen expression studies were performed on normal skin specimens obtained from different individuals. Tissue was specifically selected from the face, trunk and flexural aspect of lower limb.

To examine the expression of pemphigus vulgaris antigen, normal skin specimens from the same areas were also used.

All skin specimens were immediately frozen in liquid nitrogen and stored at -70°C until use.

Serum samples. Bullous pemphigoid studies were performed using two panels of sera from eighteen patients. Nine sera had anti-basement membrane zone antibodies in titres of 1:80 whereas the remaining nine samples had circulating bullous pemphigoid antibody titres equal to or greater than 1:160 when tested against sections of monkey oesophagus or guinea pig lip. All patients had linear deposits of IgG and/or C_3 at the basement membrane zone demonstrated by direct immunofluorescence.

Studies were also performed on twelve selected sera submitted for routine immunofluorescence tests for circulating intercellular antibodies. All serum samples were from patients diagnosed both clinically and immunopathologically as having pemphigus vulgaris. The titre of circulating pemphigus antibodies was equal to or higher than 1:80 when tested against monkey oesophagus or guinea pig lip by indirect immunofluorescence in an initial routine assay. In addition, sera from two normal individuals were used as negative controls. Samples were aliquoted and stored at -70°C.

Indirect immunofluorescence. Five micrometer (um) cryostat sections of normal skin specimens were mounted on gelatine coated slides, air dried and washed briefly in phosphate buffer saline (0.120M NaCl, 0.008M Na_2HPO_4 and 0.002M KH_2PO_4, pH7.4)

In order to avoid false positive reactions caused by blood group antibodies which can react with intercellular areas of epithelial tissues, pemphigus sera were absorbed with erythrocytes of groups A and B (1:1 vol/vol) for 30 minutes at 37°C.

Immunofluorescence testing of both bullous pemphigoid and pemphigus sera was performed according to the standard technique[173].

The cryostat sections were treated with serial dilutions of bullous pemphigoid and pemphigus vulgaris sera starting at 1:10. Phosphate buffered saline was used to dilute serum. All samples were assayed simultaneously.

The slides were placed in a moist chamber and incubated at 37°C for 30 minutes. The sections were then washed in three changes of phosphate buffered saline for 15 minutes. Rinsing in phosphate buffered saline was aimed at removing excess antibodies that are not specifically bound. The final stage of the indirect immunofluorescence assay consisted of overlying the tissue sections with fluorescein isothiocyanate (FITC) conjugated goat antihuman IgG (Dako). The protein concentration of the used conjugate was 3.7mg/ml and the Molar fluorescein to protein ratio was 2.3. The working dilution of the conjugate was 1:20. The dilution was made in phosphate buffered saline. The slides were incubated at 37°C for 30 minutes and then washed in three changes of phosphate buf-

fered saline for 30 minutes to remove unbound fluorescein-labelled antihuman IgG. The slides were drained and carefully dried around the sections.

A drop of mounting medium (glycerine/phosphate buffered saline) was placed onto each section and covered with a clean coverslip.

The slides were examined under a Leitz fluorescence microscope.

Statistical analysis. The highest dilutions of serum samples were grouped according to body site and the geometric mean was determined. A comparison between the three locations was performed using Fisher's exact test. Differences were considered significant if P value was less than 0.05.

2.1.2 Comparison of Different Epithelial Substrates Used for the Titration of Bullous Pemphigoid and Pemphigus Antibodies

Studies were performed on 36 selected sera submitted for routine immunofluorescence tests for antibodies to epithelial antigens. All serum samples included were from patients diagnosed clinically as having either bullous pemphigoid or pemphigus vulgaris. Prior to testing pemphigus sera, they were absorbed with red blood cells of groups A and B. The absorption differentiates between the

intercellular staining of the epithelium caused by pemphigus antibodies and by antibodies to blood group A and/or B antigens.

Substrates. Each serum sample was assayed on three different substrates - monkey oesophagus, guinea pig lip and human skin. These epithelial substrates are commonly used as sources of pemphigoid and pemphigus antigens in indirect immunofluorescence examination.

Experiments were performed simultaneously on all substrates.

Oesophagus and lip sections were obtained from newly sacrificed animals. Sections of normal human skin were obtained from fresh normal surgical biopsy specimens from the face. Tissue from the face was selected for its strong expression of both bullous pemphigoid and pemphigus antigens.

All tissues were immediately frozen and stored at -70°C until use.

Indirect immunofluorescence. Indirect immunofluorescence to determine antibody titres was performed by the standard technique described in section 2.1.1. All sera were tested in serial dilutions starting from 1:10. Positive and negative control sera were included with each experiment.

The positive control sera for pemphigoid and pemphigus studies were selected on the basis of reaction with comparable antibody titres on all three substrates, thus ensuring that negative or low titre reactions were not due to the poor quality of a particular substrate.

2.2 COMPARISON OF IgG SUBCLASSES AND COMPLEMENT BINDING ACTIVITY IN PATIENTS WITH BULLOUS PEMPHIGOID AND PEMPHIGUS VULGARIS

The distribution of IgG subclasses in the skin and serum of patients with pemphigoid and pemphigus was investigated. The diagnosis of bullous pemphigoid was confirmed by direct immunofluorescence of perilesional skin demonstrating anti-basement membrane zone antibodies and complement. The diagnosis of pemphigus was established by the detection of IgG antibodies in the intercellular areas of the epidermis by direct immunofluorescence.

Tissue. Perilesional skin from 17 patients with bullous pemphigoid and 10 patients with pemphigus vulgaris were included in the study. All skin specimens were obtained during active disease.

Serum. Samples from 25 bullous pemphigoid patients and 17 pemphigus vulgaris patients were included in this investigation.

Normal human skin and serum from healthy individuals served as controls.

Substrate. Monkey oesophagus was used as a substrate for indirect immunofluorescence studies. Fresh specimens were stored at -70°C until used. Prior to testing, a block of the substrate was mounted and 5um sections were cut on a cryostat.

2.2.1 Direct Immunofluorescence[173]

Five um cryostat sections of perilesional pemphigoid and pemphigus skin were first tested for IgG and C_3 deposits. The third component of complement was detected by FITC-conjugated rabbit antihuman C_3 complement (Dako). The protein concentration of the conjugate was 4.4mg/ml and the Molar fluorescein to protein ratio was 2.3. The working dilution of the conjugate was 1:20. The reagent was diluted in phosphate buffered saline.

The slides were placed in a moist chamber and incubated for 30 minutes at 37°C. The slides were then washed in several changes of phosphate buffered saline for 30 minutes. This immunofluorescence technique was also used to determine the distribution of IgG subclasses in pemphigoid and pemphigus skin.

The frozen tissue sections were overlaid with mouse monoclonal antibodies against the human IgG subclasses G_1, G_2, G_3 and G_4 (Sigma). The antibodies were produced by the fusion of mouse myeloma cells and spleen cells from an

immunized mouse. All the IgG subclasses antibodies used were conjugated to FITC. A working dilution of 1:50 in phosphate buffered saline was used.

Antihuman IgG_1 - Clone No. 8C/6-39. The specific immunoglobulin concentration = 4.5mg/ml. The Molar fluorescein to protein ratio was 6.0.

Antihuman IgG_2 - Clone No. HP-6014. The Molar fluorescein to protein ratio was 4.0.

Antihuman IgG_3 - Clone No. HP-6050. The Molar fluorescein to protein ratio was 3.6.

Antihuman IgG_4 - Clone No. HP-6025. The Molar fluorescein to protein ratio was 4.1.

The protein concentration of the monoclonal antibodies IgG_2, IgG_3 and IgG_4 is not available.

There is no cross reactivity between the different IgG subclasses. The specificity of each clone has been determined by several immunological methods[174].

2.2.2 Indirect Immunofluorescence

Pemphigoid and pemphigus sera, diluted 1:10, were first screened for the presence of IgG against the basement membrane zone and intercellular areas using polyclonal goat antihuman IgG. Subsequently, indirect immunofluorescence studies to assay the IgG subclasses of anti-basement membrane zone and intercellular antibodies were performed. Direct and indirect immunofluorescence

results were graded according to the intensity of fluorescence: (++) = strong positive; (+) = positive; (-) = negative staining.

Preliminary testing of pemphigoid and pemphigus skin and sera showed no demonstrable IgG_2. Therefore, this particular subclass was not sought in subsequent experiments.

2.2.3 Indirect Complement Immunofluorescence

To examine the relationship between IgG subclasses, distribution in pemphigoid and pemphigus antibodies, and their capability to fix complement, an indirect complement immunofluorescence technique was used. The investigation included all pemphigoid and pemphigus serum samples for which the distribution of IgG subclasses has been determined.

The indirect complement immunofluorescence method used, which measures the avidity with which antibodies fix complement, was based on a modification of the method described by Beutner et al.[173].

The procedure consists of three stages. The first stage is similar to the initial step of classical indirect immunofluorescence. Normal human skin was used as a substrate. Cryostat sections were overlaid with patients' sera at a dilution of 1:10. In the second stage the sections were incubated with a source of complement. Serum

collected from healthy individuals was used as a source of complement. Normal serum was stored at -70°C and used within two weeks.

Complement fixing IgG antibodies that reacted with antigen in the first stage activate complement in the second stage to generate numerous C_3 molecules which are deposited at the antigen antibody binding sites. The final stage consisted of incubating the sections with FITC conjugated rabbit antihuman C_3 antibody.

All incubations were for 30 minutes at 37°C. The slides were washed in phosphate buffered saline for 15 minutes between incubations. Appropriate controls were included with each experiment.

2.1 IMMUNOFLUORESCENCE - SPLIT SKIN AND WESTERN IMMUNOBLOTTING STUDIES

2.3.1 Indirect Immunofluorescence Using Sodium Chloride-Split Skin

Sera. Serum samples were obtained from 27 patients with bullous pemphigoid. Sera were collected during active disease. Immunopathologic studies showed deposition of IgG and/or C_3 along the basement membrane zone of the skin by direct immunofluorescence. Circulating anti-

basement membrane zone antibodies were detected by standard indirect immunofluorescence technique on cryostat sections of monkey oesophagus or guinea pig lip. Antibody titres ranged between 1:40 and 1:320. A serum sample from a patient with the diagnosis of epidermolysis bullosa acquisita was also included. Direct immunofluorescence of perilesional skin from this patient showed intense linear deposits of IgG and C_3 at the basement membrane zone. Electron microscopy of a skin biopsy showed a cleavage beneath the lamina densa. Circulating IgG anti-basement membrane zone antibodies were positive at a titre of 1:160 when tested on cryostat sections of normal human skin. Normal sera from 7 healthy individuals were used as negative controls.

Skin. Several pieces of normal human skin were obtained from the flexor aspect of the forearm and leg from different individuals with no evidence of blistering or autoimmune disease. Half of one specimen was quickly frozen in liquid nitrogen and stored at -70°C. It was used to compare the sensitivity of intact and separated skin for the detection of anti-basement membrane zone antibodies. Excess fat was removed from all specimens, after which they were washed in phosphate buffered saline and treated with 1M sodium chloride according to the method of Scaletta et al.[175] as follows: the samples were incubated for 48-96 hours in freshly prepared 1M sodium chloride at 4°C or at room temperature with constant

agitation. The sodium chloride solution was changed every 24 hours. At the end of the incubation period the specimens were placed with the dermis side down. The epidermis was then gently separated from the dermis using forceps. Alternatively, the epidermis may be carefully dislodged from the dermis by gentle traction to the skin. Specimens were frozen and stored at -70°C until use.

Immunoreagents.

Mouse anti-type IV collagen (Dako). The protein concentration of this antibody was 4.05mg/ml. It was used at a dilution of 1:50.

Mouse anti-type VII collagen. This monoclonal antibody was obtained from Serotec. The antibody specifically reacts with the carboxyl terminus of type VII collagen. It was used at a dilution of 1:10.

FITC-conjugated rabbit anti-mouse immunoglobulins (Dako). The protein concentration of this reagent was 3.1mg/ml and the Molar fluorescein/protein ratio was 2.3. The conjugate was used at a dilution of 1:20 in phosphate buffered saline.

Indirect immunofluorescence. The procedure was performed using 5um cryostat sections of intact and separated skin. Testing was carried out on intact skin and sections from two different specimens of separated skin to localize type IV collagen and type VII collagen and to confirm the ultrastructural level of separation. Bullous pemphigoid, epidermolysis bullosa acquisita and control sera were ex-

amined at a dilution of 1:10 on intact and split skin. The epidermolysis bullosa acquisita serum and 12 bullous pemphigoid sera were also reacted against two other specimens of separated skin. In addition, the epidermolysis bullosa acquisita sample and 8 pemphigoid sera were simultaneously tested in serial dilutions on intact and split skin.

2.3.2 Direct Immunofluorescence of Sodium chloride-Separated Biopsy Specimens

Perilesional skin biopsy specimens from seven patients with bullous pemphigoid and a patient with epidermolysis bullosa acquisita were separated at the dermal epidermal junction by sodium chloride. All patients had deposits of IgG and C_3 at the basement membrane zone demonstrated by routine direct immunofluorescence. The specimens were obtained one year previously and had been frozen and stored at -70°C. They were thawed and incubated in 1M sodium chloride solution as described above. Direct IgG and C_3 immunofluorescence of the separated biopsies was performed using the standard technique. The ultrastructural level of split was confirmed by immunofluorescence mapping using monoclonal antibodies against type IV and type VII collagen.

2.3.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Immunoblotting Studies

This study included seven of the 27 pemphigoid sera initially examined on split skin. All samples gave an epidermal staining pattern. An epidermolysis bullosa acquisita serum which produced a dermal pattern of staining was also tested. All sera had a titre equal to or greater than 1:160 when tested on salt-split skin. Normal human serum from healthy individuals was used as a control.

In addition, sera from patients with other dermatoses also served as controls. This group included two patients with psoriasis, two with generalized eczema, five with pemphigus vulgaris and two with pemphigus foliaceus. All pemphigus sera contained circulating intercellular antibodies at titres greater than 1:160.

Epidermal extraction. The epidermal extract was prepared using normal human skin obtained from surgical specimens of breast. The skin was cut into small pieces 0.5 x 0.5cm or strips 5 x 0.5cm and incubated in a fresh solution of 1M sodium chloride at 4°C for 72-96 hours. The solution also included 1mM of phenyl methyl sulfonyl fluoride (PMSF) and 2mM of ethylenediaminetetra acetic acid (EDTA). The reactivity of separated skin was tested by indirect immunofluorescence using epidermolysis bullosa acquisita and bullous pemphigoid sera. After dermal epidermal separation the bullous pemphigoid antigen was

extracted from the epidermis following the procedure of Stanley et al.[142]. Approximately 25cm² of epidermis was added to 2ml of extraction buffer composed of 62.5mM Tris HCl pH 6.8, 2% w/v SDS, 2mM PMSF, 10mM EDTA and 0.3M mercaptoethanol, and incubated at room temperature for one hour. The epidermis was then homogenized using a motor-driven homogenizer for 60 seconds (4 x 15s) at room temperature. The homogenate was then centrifuged at 13500 rpm (9500 x g) for 20 min. The supernatant was collected, divided into aliquots and stored at -70°C until use.

Dermal extraction. Dermal extracts were prepared using a modification of the method described by Woodly et al.[146]. The dermis was extracted for one hour at room temperature with 12.5mM tris HCl, pH 6.8, containing 8M urea, 2%(w/v) SDS, 0.3M mercaptoethanol, 1mM PMSF and 2mM EDTA. The preparation was then vortexed for 15 min. at room temperature and then centrifuged at 13500 rpm (9500 x g) for 20 min. The supernatant was collected and stored at -70°C until it was used for electrophoresis.

Protein content estimation. The protein concentration in epidermal and dermal extracts was estimated by Lowry's assay[176].

Reagents: Reagent A, 2% Na_2CO_3 in 0.1M NaOH

Reagent B, 0.5% $CuSO_4.5H_2O$ in sodium citrate

Reagent C, alkaline copper solution - mix 50ml of solution A with 1ml of reagent B

Reagent D, prepared by adding equal volumes of Folin's solution and distilled water.

Preparation:

0.1ml of epidermal or dermal extract sample, diluted 1:50, and 0.1ml 1M NaOH were placed in test tubes. Distilled water was added to a final volume of 1ml.

Tubes containing 0, 0.02, 0.04, 0.06, 0.08 and 0.1ml of bovine serum albumin (1mg/ml) were run in parallel to provide a standard curve. Two ml. of reagent C were added to all samples and standards. The tubes were vortexed for a few seconds and left at room temperature for 10 min. Finally 0.2ml of reagent D was added rapidly and the tubes briefly mixed on a vortex mixer, then left at room temperature for 30 min. Absorbances were measured at 550nm.

SDS-PAGE of epidermal and dermal extracts. This was performed according to the method of Laemmli[177]. The composition of the running and stacking gels is indicated in Table 1.

Gels were cast in a cassette consisting of two glass plates (160 x 180 x 3mm) kept apart with 2mm spacers and held together by two clamps.

The running gel (125 x 145 x 2mm) was poured, overlaid with distilled water and allowed to polymerize for approximately 30 min. After removing the overlay solution, the stacking gel (35 x 145 x 2mm) was cast onto the

Table 1

THE COMPOSITION OF THE RUNNING AND STACKING GELS

	Component	Running Gel/50 ml	Stacking Gel/10 ml
*	Acrylamide stock	8.4 ml (5.0%) 10 ml (6.0%)	1.7 ml (5.0%)
	1.5M Tris HCl, pH 8.8	12.5 ml	
	0.5M Tris HCl, pH 6.8		1.25 ml
	Distilled water	28.1 ml (5.0%) 26.5 ml (6.0%)	6.8 ml
	10% (w/v) SDS	0.5 ml	0.1 ml
	10% (w/v) Ammonium persulfate	0.5 ml	0.1 ml
**	TEMED	0.04 ml	0.01 ml

* The acrylamide stock solution contained 29.2g acrylamide and 0.8g N,N' - Methylene bis acrylamide in 100 ml of distilled water.

** TEMED = N,N,N',N' - tetramethylenediamine

surface of the resolving gel. A clean Teflon comb was immediately inserted into the stacking gel solution. The gel was allowed to polymerize at room temperature.

Samples of epidermal and dermal proteins were prepared for electrophoresis by incubating in sample buffer at 100°C for 3 min. The composition of sample buffer is shown in Table 2.

Standard protein markers were also run for calibration. A mixture of high molecular weight standards was purchased from Sigma. The mixture contained the standards - Myosin (205KD), B-Galactosidase (116KD), Phosphorlase B (97KD) and Bovine Albumin (66KD). It was prepared in the appropriate sample buffer and boiled before loading on gels.

When polymerization of the stacking gel was complete (approximately 30 min.), the Teflon comb was removed and up to 75ug of epidermal or dermal proteins were loaded per well.

The whole cassette was then mounted in the electrophoresis apparatus. Electrophoresis was performed at 50mA for 4-5 hours. The electrophoresis was stopped when the dye front had travelled at least 100mm into the resolving gel.

The gel running buffer, which was added to both upper and lower reservoirs, contained 0.192M glycine, 0.1% (w/v) SDS in 0.25M Tris HCl (pH 8.3).

Table 2

COMPOSITION OF SAMPLE BUFFER

Component	Volume
0.5 M Tris HCl, pH 6.8	1.25 ml
Distilled water	4 ml
Glycerol	2 ml
10% (w/v) SDS	2 ml
2-Mercaptoethanol	0.5 ml
0.05% (w/v) Bromophenol blue	0.25 ml

Western blotting. Immediately after electrophoresis, proteins from SDS-PAGE gels were transferred to nitrocellulose sheets following the method of Towbin[178]. Transfer was carried out at room temperature using a BioRad trans-blot cell. The transfer (Towbin's) buffer contained 25mM Tris HCl, 192mM glycine and 20% (v/v) methanol at pH 8.3.

Transfer was performed at 80mA for approximately 18 hours.

Gels were then transferred to a tray containing Coomassie blue to monitor the disappearance of proteins from gels.

Staining with Coomassie blue. Coomassie blue is a staining reagent used in the procedure of SDS-PAGE and Western blotting to visualize protein bands on SDS gels and to establish the efficiency of transfer of proteins from gels to nitrocellulose filters.

The gel was immersed in the staining solution and left at room temperature for 3 hours. The stain was then removed and the gel de stained over a period of 4-6 hours in 5% methanol (v/v) and 7.5% (v/v) acetic acid.

Staining with Ponceau S. Proteins may be stained directly on the nitrocellulose membrane with Ponceau S stain to establish that the electrophoretic transfer of proteins has taken place and to determine the location of molecular weight markers. The staining procedure was performed immediately after the nitrocellulose sheet was removed from the blotting apparatus. It was then trans-

ferred to a tray containing a solution of Ponceau S (Sigma) and incubated at room temperature for 10 min. with gentle agitation. It was then briefly washed in distilled water to visualize lanes and protein bands.

The molecular weight marker lanes were cut off before proceeding to the immunostaining stage.

Immunostaining. The nitrocellulose sheet from the Western blot was incubated for one hour at room temperature in 5% (w/v) non-fat dried milk to block free protein binding sites and then washed briefly with phosphate buffered saline.

The nitrocellulose filter was cut lengthwise into 0.5cm strips corresponding to each sample application lane of the acrylamide gel.

The strips were placed in a tray and each incubated with 1-2ml of patient's or control serum diluted 1:5 with 0.05% Tween 20 in phosphate buffered saline pH 7.4. The incubation was at 37°C for two hours with gentle agitation.

The nitrocellulose strips were then washed with phosphate buffered saline-Tween 20 3 times for 5-10min. Each strip was then incubated for one hour at 37°C with 1:200 dilution of a peroxidase conjugated goat antihuman IgG (SAPU) in washing buffer.

After 3 washes with phosphate buffered saline-Tween 20 the strips were developed at room temperature using 4-chloronaphthol. Colour development was complete in 20-30 min.

2.4 THE SPECIFICITY AND SENSITIVITY OF ENDOMYSIAL ANTIBODIES

Serum samples were collected from twenty patients with dermatitis herpetiformis. Direct immunofluorescence was performed to detect tissue bound IgA antibodies. Five patients had been on a gluten-free diet for at least six months. The remaining fifteen patients were on a normal diet. A control group consisting of 20 patients with pemphigus and 20 patients with bullous pemphigoid was included. The serum samples were stored at -70°C until processing. The presence of endomysial antibodies was sought by standard indirect immunofluorescence. Testing was performed on 5um cryostat sections of monkey oesophagus. The sections were incubated with patients' sera starting at 1:2.5 dilution. A positive control serum was included in each experiment. After serum treatment the sections were washed in phosphate buffered saline and incubated with fluorescein conjugated goat antihuman IgA. The protein concentration of the conjugate was 8.8mg/ml

and the molar fluorescein:protein ratio was 3.1. The reagent was used at a dilution of 1:20 in phosphate buffered saline.

3. RESULTS

3.1 THE REACTIVITY OF BULLOUS PEMPHIGOID AND PEMPHIGUS ANTIBODIES ON DIFFERENT SUBSTRATES

Normal human skin has been regarded by some authors as an inferior substrate for the titration of circulating anti-basement membrane zone and anti-intercellular antibodies in pemphigoid and pemphigus[173]. However, this view has not taken into account the differences in the distribution of bullous pemphigoid and pemphigus antigens at various areas of the body.

3.1.1 Variations in the Distribution of Bullous Pemphigoid and Pemphigus Antigens in Normal Skin

To study the expression of the bullous pemphigoid antigen at different body sites a panel of nine sera (samples nos. 1-9) from patients with bullous pemphigoid was reacted with three skin specimens from the face, trunk and limb. The results showed variations in the maximum dilution of bullous pemphigoid sera giving positive reactions when tested against skin from different regions of the body. The titre of bullous pemphigoid antibodies

ranged from 0-160. Eight of the nine samples examined had antibodies to bullous pemphigoid antigen in normal skin when tested on sections from the three skin specimens. One of the nine sera failed to demonstrate bullous pemphigoid antigen in trunk tissue (Table 3).

The greatest expression of bullous pemphigoid antigen, as estimated from the end point titre of bullous pemphigoid antibody, was with skin from the face and limb. In contrast, high concentrations of bullous pemphigoid antibodies were required to detect the antigen in trunk tissue, indicating that only small amounts were present.

These studies were repeated using another panel of high titre bullous pemphigoid sera (samples nos. 10-18).

The results of testing the additional nine sera (Table 3) showed that the greatest expression of bullous pemphigoid antigen was also with skin obtained from the face and limb. The antibody level in these samples ranged between 160-640 when tested against facial skin. Slightly lower titres were observed with sections from limb skin. Most sera showed a titre above 1:80. Titres of the same sera ranged from 1:10 to 1:160 when skin from the trunk was used as a substrate. In most instances low dilutions were required to demonstrate bullous pemphigoid antigen with the majority of reactions at or below 1:40.

Statistical evaluation showed a significant difference between the geometric mean of all end point titres obtained on facial skin and trunk skin. Similarly, a

Table 3

THE EXPRESSION OF BULLOUS PEMPHIGOID ANTIGEN IN SKIN OBTAINED FROM VARIOUS LOCATIONS

Serum No.	Facial Skin	Trunk Skin	Limb Skin
1	1:80	1:20	1:40
2	1:80	0	1:40
3	1:80	1:40	1:40
4	1:80	1:10	1:40
5	1:40	1:20	1:40
6	1:80	1:40	1:80
7	1:160	1:160	1:160
8	1;80	1:20	1:80
9	1:80	1:40	1:80
10	1:320	1:20	1:160
11	1:160	1:10	1:80
12	1:640	1:40	1:80
13	1:160	1:40	1:160
14	1:640	1:40	1:640
15	1:160	1:40	1:640
16	1:320	1:160	1:320
17	1:160	1:20	1:160
18	1:160	1:10	1:40

significant difference was detected between the geometric mean of titres on limb skin and trunk skin ($P<0.05$, Fisher's exact test). There was no significant difference between titres on facial and limb skin (Figure 5). These results showed that variations between tissue reactivity may reflect differences in antigen expression at different sites on the body.

Pemphigus vulgaris. Studies to determine whether the regional differences in antigen expression is a phenomenon specific to bullous pemphigoid antigen or involve the pemphigus vulgaris antigen were also performed. The expression of this antigen was also estimated by indirect immunofluorescence using skin specimens from the face, trunk and limb. Each specimen was reacted with serial dilutions of twelve pemphigus vulgaris sera (Table 4). The expression of the antigen in different body regions was estimated from the end point titre obtained when a serum sample was tested against sections from a particular anatomic area.

In contrast to bullous pemphigoid, the expression of pemphigus antigen in normal skin from three different areas of the body was similar. The difference was limited to one dilution. Figure 6 shows a comparison between the geometric mean of titres on specimens from the different body regions. There was no significant difference between the mean of end point titres obtained on different sites. There was some correlation between the patterns of antigen

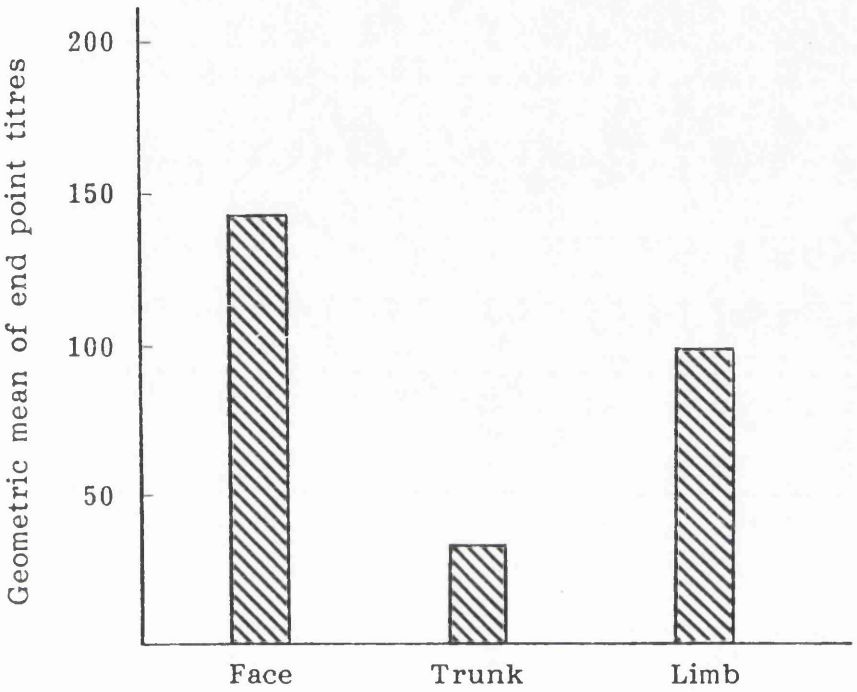


Figure 5. Geometric means of all bullous pemphigoid sera end point titres on different skin specimens.

Table 4

QUANTITATIVE EXPRESSION OF PEMPHIGUS VULGARIS ANTIGEN IN SKIN OBTAINED FROM DIFFERENT SITES

Serum No.	Facial Skin	Trunk Skin	Limb Skin
1	1:160	1:80	1:80
2	1:640	1:640	1:320
3	1:80	1:80	1:80
4	1:80	1:80	1:80
5	1:320	1:320	1:160
6	1:160	1:160	1:160
7	1:320	1:320	1:320
8	1:80	1:80	1:160
9	1:160	1:160	1:320
10	1:160	1:160	1:160
11	1:80	1:40	1:80
12	1:80	1:80	1:40

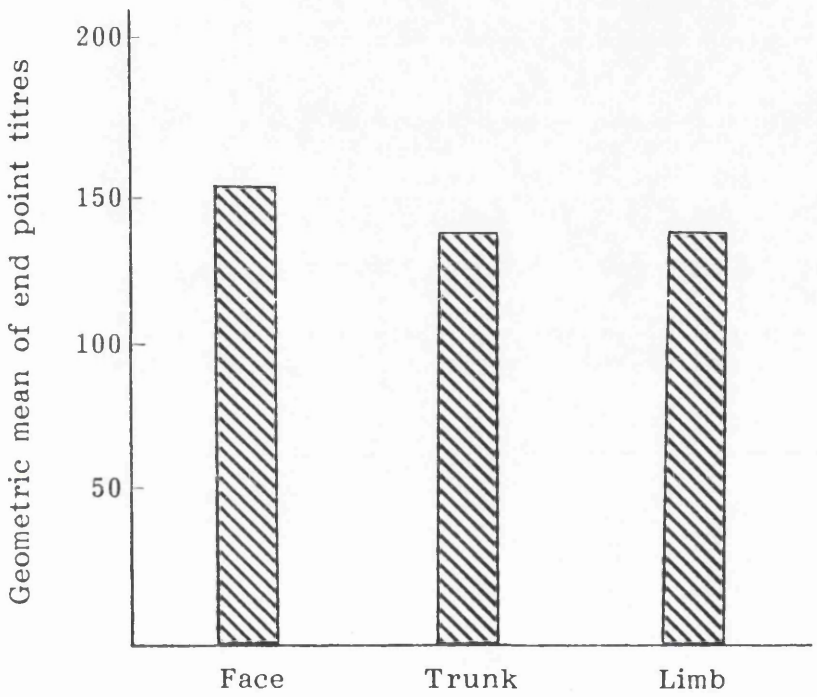


Figure 6. Geometric means of all pemphigus vulgaris sera end point titres on different skin specimens.

expression in pemphigoid and pemphigus. Marked concentrations of both antigens were found in facial and limb skin. Conversely, the expression of pemphigus vulgaris antigen was high in trunk tissue which showed much less expression of the bullous pemphigoid antigen.

The results therefore indicate a difference in the distribution between bullous pemphigoid and pemphigus vulgaris antigens with the latter being more uniformly expressed over the body.

3.1.2 The Sensitivity of Normal Skin, Primate Oesophagus and Guinea Pig Lip for the Determination of Circulating Antibodies

Studies were performed on 21 sera from patients with bullous pemphigoid and 15 sera from pemphigus vulgaris patients. Indirect immunofluorescence testing was carried out using three epithelial substrates - monkey oesophagus, guinea pig lip and normal human skin. Tissue from the face was selected for its strong expression of both pemphigoid and pemphigus antigens.

All samples were tested simultaneously on all substrates to exclude variations in technique which may influence the assessment of reactions and antibody titre levels. Some sera showed comparable reactivity on all three antigenic substrates; however, differences in titre were encountered with many of the samples tested.

Bullous pemphigoid. The results of the affinity of bullous pemphigoid antibodies for the different antigenic substrates are summarized in Table 5. In spite of the differences in reactivity, all sera yielded a positive reaction on sections from the three epithelial sources. Antibody titres obtained with facial skin were generally lower than those obtained using animal substrates. In the 21 sera studied, antibody levels ranged between 1:40 and 1:160 when tested against normal skin and between 1:40 and 1:640 when monkey oesophagus and guinea pig lip were used as substrates.

The pattern of reactivity of bullous pemphigoid sera studied is illustrated in Figure 7. Seventeen (81%) of the 21 assays performed with monkey oesophagus were positive in titres equal to or greater than 1:160 compared to 13 (62%) when guinea pig lip was used as a substrate and 9 (43%) of 21 with normal skin. None of the sera tested reacted more strongly on facial skin than on the animal substrates. Two samples gave identical results on all three substrates. Conversely, the end point titre on monkey oesophagus exceeded that on normal skin by one to two dilutions in 15 samples and by three dilutions in one case.

Analysis of the binding of bullous pemphigoid antibodies to each of the two animal substrates showed that antibody titres were identical in 8 of the 21 samples tested. Four sera displayed higher titres on guinea pig

Table 5

THE REACTIVITY OF BULLOUS PEMPHIGOID SERA ON DIFFERENT SUBSTRATES

Serum	Facial Skin	Monkey Oesophagus	Guinea Pig Lip
1	1:40	1:80	1:40
2	1:40	1:160	1:80
3	1:80	1:160	1:80
4	1:80	1:160	1:80
5	1:40	1:80	1:40
6	1:40	1:160	1:40
7	1:160	1:160	1:160
8	1:40	1:80	1:80
9	1:160	1:320	1:320
10	1:80	1:160	1:160
11	1:80	1:160	1:160
12	1:40	1:40	1:40
13	1:80	1:160	1:160
14	1:80	1:640	1:160
15	1:160	1:160	1:320
16	1:160	1:160	1:320
17	1:160	1:640	1:640
18	1:160	1:160	1:640
19	1:160	1:160	1:320
20	1:160	1:320	1:160
21	1:160	1:320	1:160

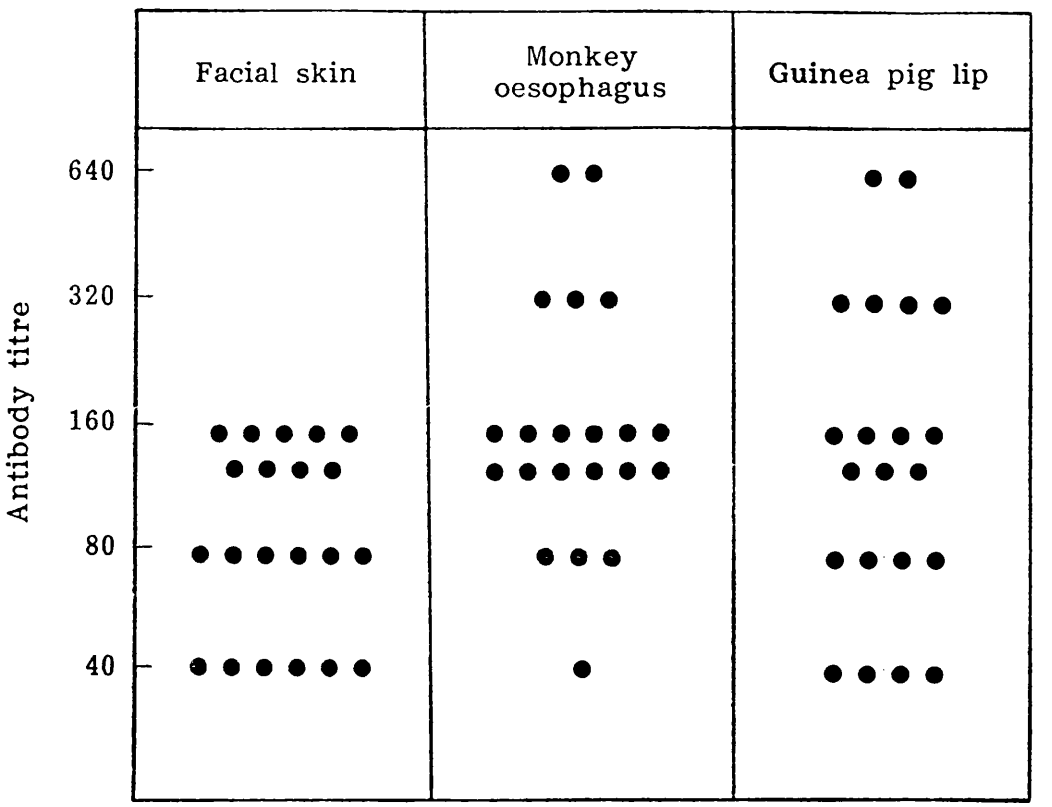


Figure 7. Comparison of bullous pemphigoid antibodies in 21 sera reacted with facial skin, monkey oesophagus and guinea pig lip.

lip whereas 8 sera yielded higher end point titres on monkey oesophagus. The titre difference ranged between one and two doubling dilutions. Figure 8, A-C, illustrates the characteristic staining of bullous pemphigoid antibodies on different substrates.

Pemphigus vulgaris. Serum samples from 15 patients with pemphigus vulgaris were concurrently tested on normal human skin, monkey oesophagus and guinea pig lip to determine the most sensitive substrate for the detection of intercellular antibodies by indirect immunofluorescence.

The study showed variations in antibody levels between human skin and animal tissue (Table 6). The staining reactions observed with monkey oesophagus and guinea pig lip were similar. On both substrates the antibodies produced a fine sharp linear fluorescence. However, the intercellular staining on human skin sections was more diffuse and, in some cases, more difficult to interpret.

The titre of intercellular antibodies ranged from 1:40 to 1:640 when tested against normal skin and 1:40 to 1:1280 when guinea pig lip and 1:40 to 1:2560 when monkey oesophagus was used as a substrate (Figure 9). The majority of sera displayed lower titres when reacted with sections of normal skin. Five (33%) of 15 assays performed with facial skin were positive in titres equal to or greater than 160 compared to 11 (73%) when guinea pig lip and 13 (87%) when monkey oesophagus were used in the indirect immunofluorescence assay. None of the 15 sera

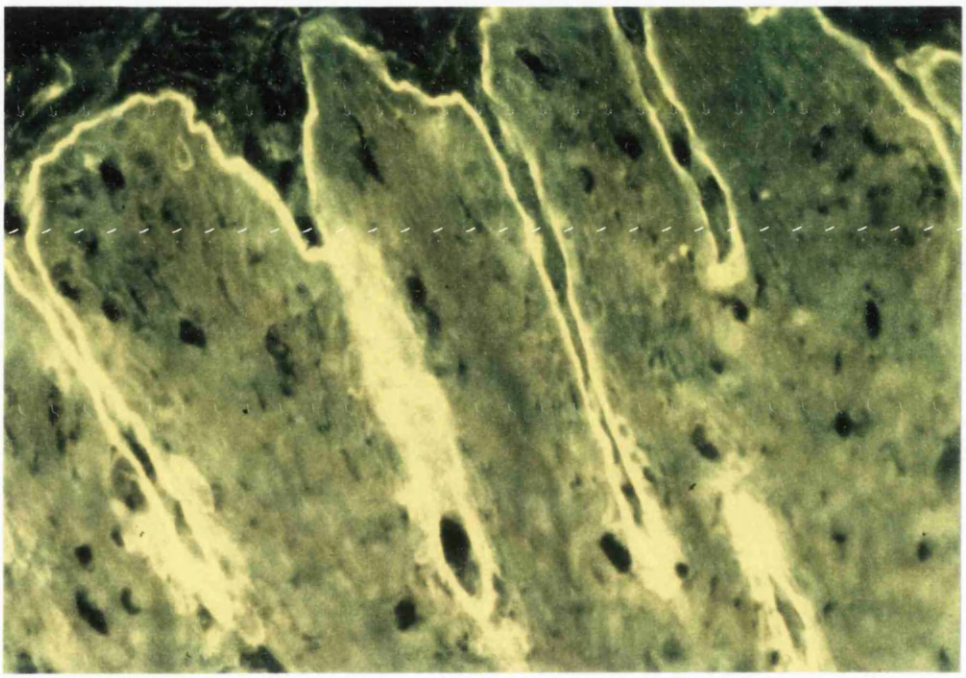


Figure 8. Basement membrane zone staining by bullous pemphigoid antibodies on different epithelial substrates.

A. Monkey oesophagus

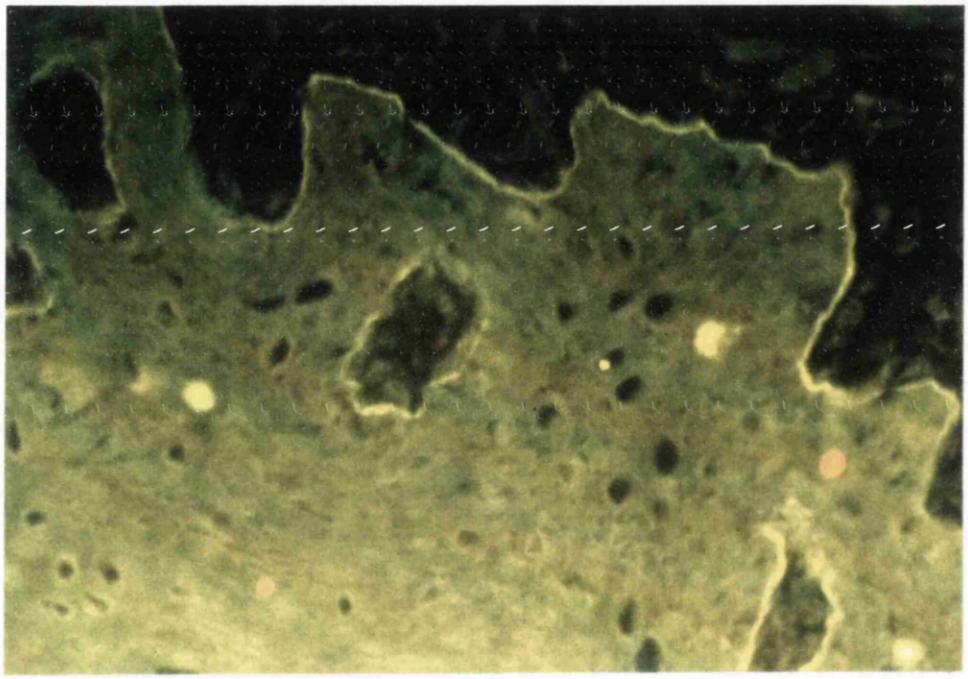


Figure 8 (continued). B. Guinea pig lip

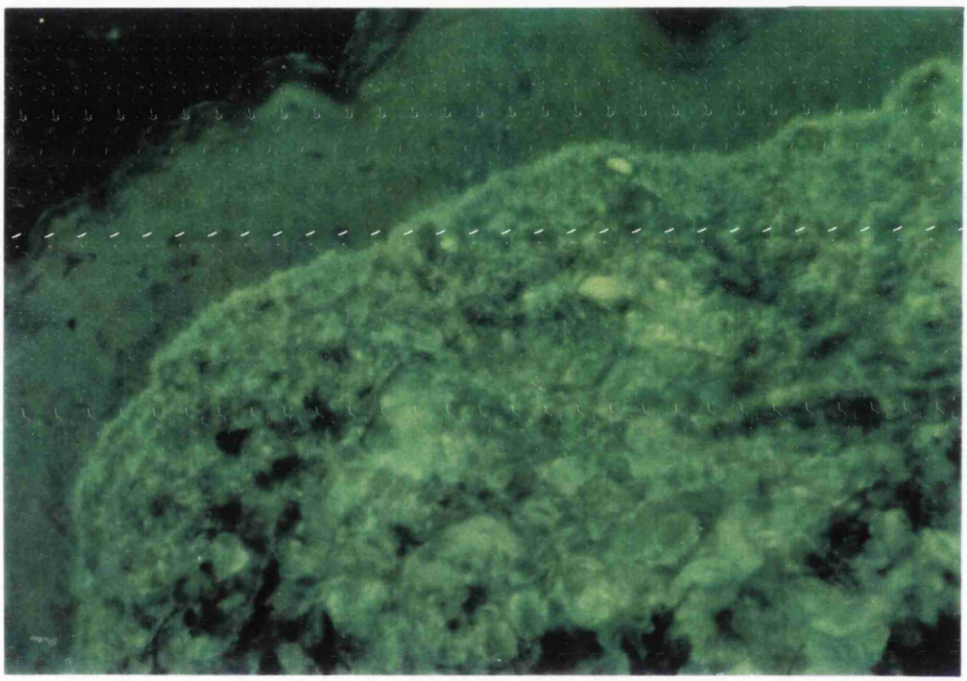


Figure 8 (continued). C. Human skin.

Table 6

THE REACTIVITY OF PEMPHIGUS SERA
ON DIFFERENT SUBSTRATES

Serum	Facial Skin	Monkey Oesophagus	Guinea Pig Lip
1	1:40	1:80	1:80
2	1:40	1:40	1:80
3	1:40	1:160	1:80
4	1:320	1:320	1:320
5	1:80	1:320	1:320
6	1:40	1:320	1:160
7	1:80	1:320	1:320
8	1:640	1:2560	1:1280
9	1:80	1:160	1:320
10	1:160	1:1280	1:640
11	1:80	1:160	1:40
12	1:80	1:160	1:160
13	1:160	1:320	1:160
14	1:160	1:320	1:320
15	1:80	1:160	1:160

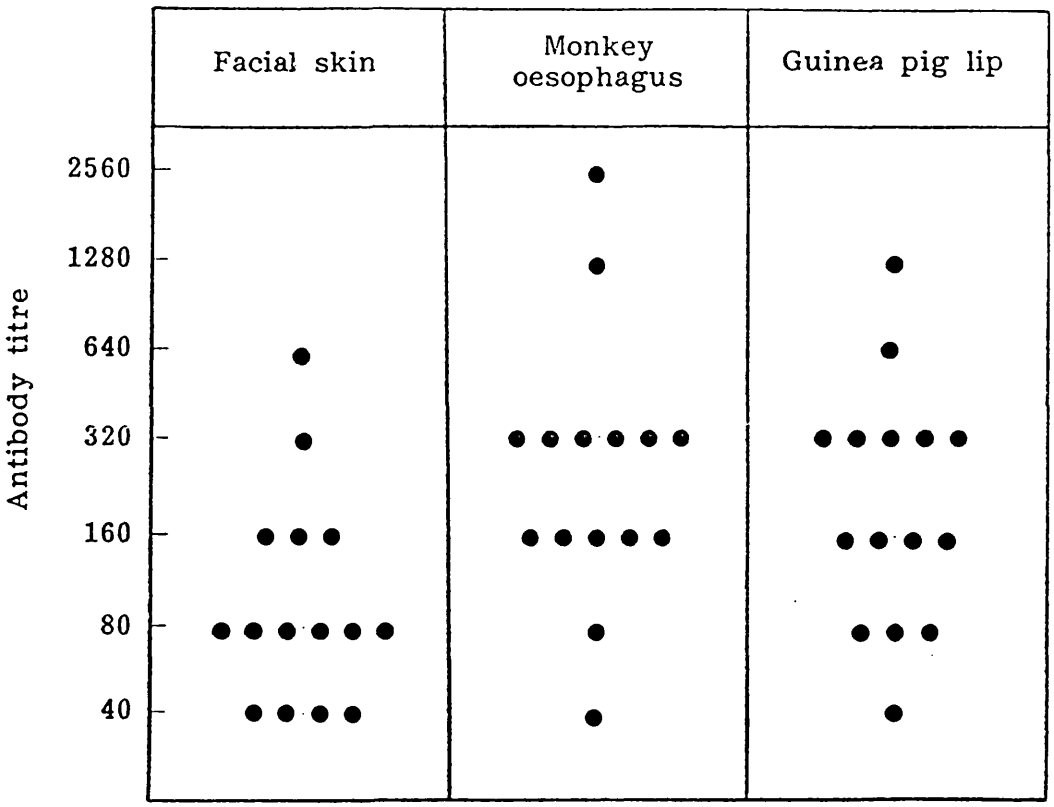


Figure 9. The reactivity of pemphigus sera with facial skin, monkey oesophagus and guinea pig lip.

tested gave a higher antibody titre on normal skin than on monkey oesophagus or guinea pig lip. One sample yielded identical results on normal skin and the animal substrates. In the remaining 14 sera the end point titre on either monkey oesophagus or guinea pig lip exceeded that on normal skin sections by up to three doubling dilutions.

The intercellular antibody titres were identical on sections from both animal substrates in 7 of the 15 sera examined.

Six samples gave a higher titre on monkey oesophagus than on guinea pig lip by one or two doubling dilutions, whereas two samples were more reactive with guinea pig lip by one dilution.

Figure 10, A-C, illustrates the characteristic staining pattern of pemphigus antibodies on various substrates.

3.2 THE DISTRIBUTION OF TISSUE BOUND AND CIRCULATING IgG SUBCLASSES IN PEMPHIGOID AND PEMPHIGUS

3.2.1 IgG Subclasses in Bullous Pemphigoid

Skin subclass distribution. A summary of the subclass distribution of anti-basement membrane zone antibodies in perilesional skin biopsies from patients with pemphigoid is shown in Table 7.

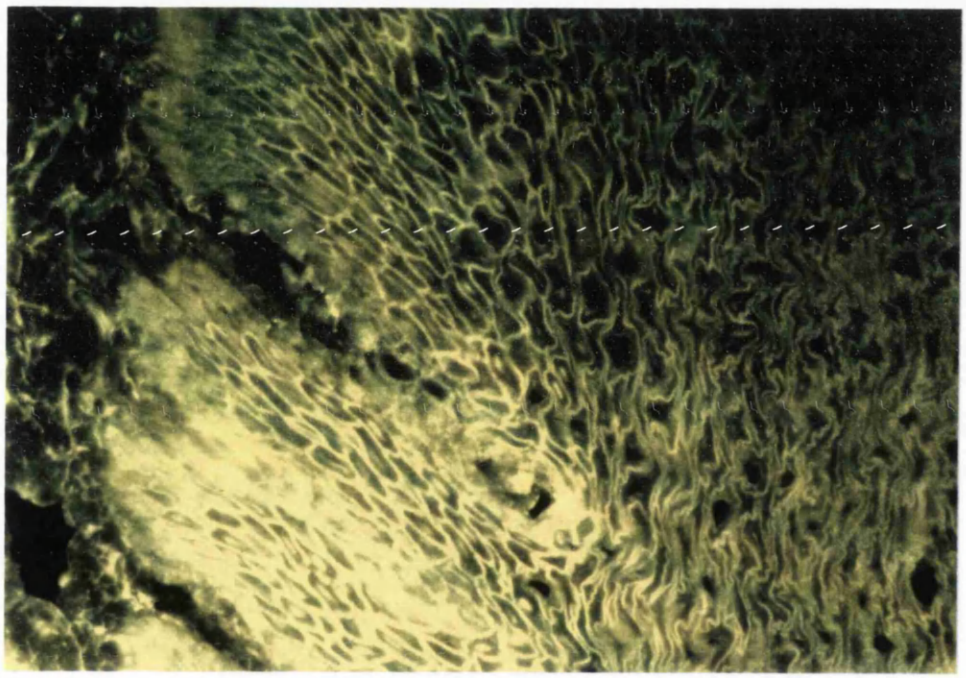


Figure 10. Intercellular staining by pemphigus vulgaris antibodies on different epithelial substrates.

A. Monkey oesophagus.

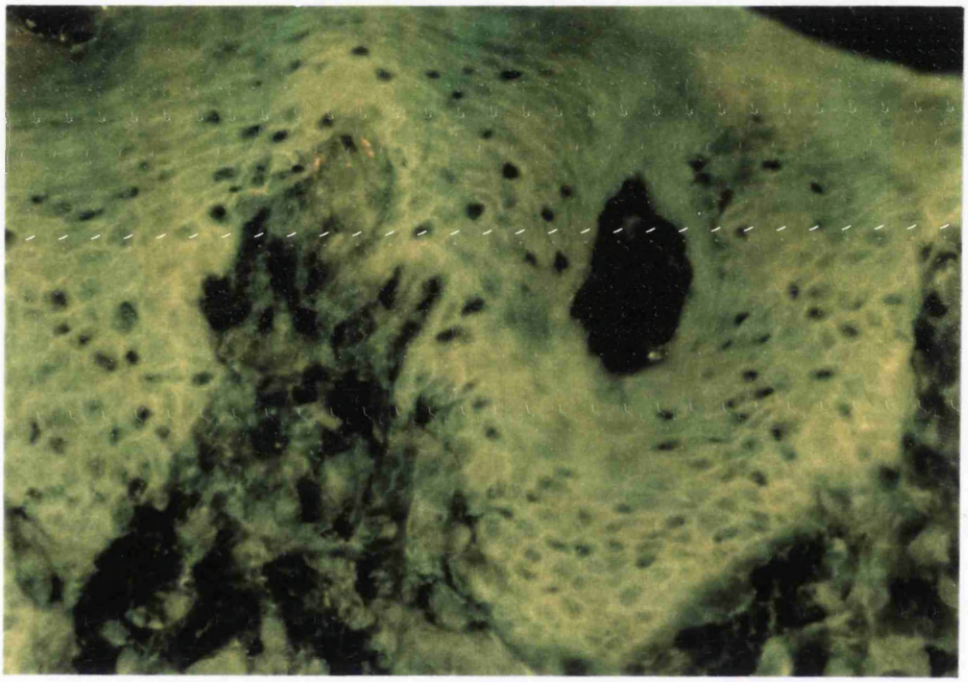


Figure 10 (continued). B. Guinea pig lip.

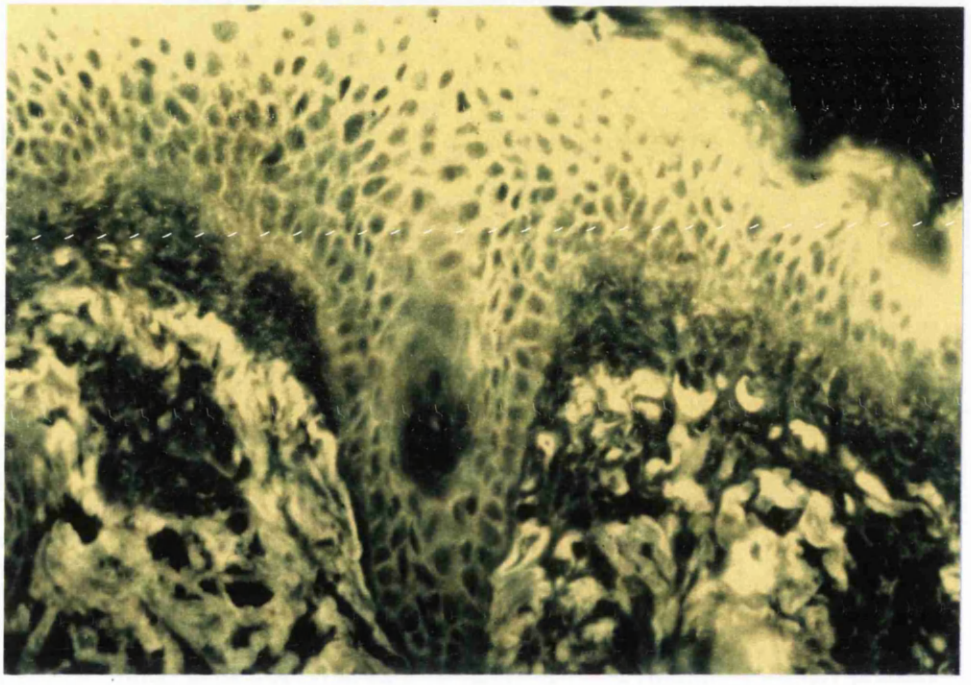


Figure 10 (continued). C. Human skin

Table 7

IgG SUBCLASSES OF TISSUE BOUND ANTIBODIES
IN BULLOUS PEMPHIGOID

Fluorescence	IgG	IgG_1	IgG_3	IgG_4	C_3
Negative	0	7	14	2	1
Positive	0	8	3	6	0
Strong Positive	17	2	0	9	16
Total Positive	17	10	3	15	16
Percent Positive	100	59	18	88	94

Deposits of IgG were present in all 17 cases studied, 15 of which contained IgG_4, 10 IgG_1, and 3 IgG_3. In only two cases was IgG_4 autoantibody not detected. However, both cases were positive for IgG_1, and in one of the two cases IgG_3 was also demonstrated (Figure 11, A-C).

In 5 (29%) of 17 biopsies IgG_4 was the only IgG subclass detected, whereas IgG_1 was the only subclass observed in one (6%) of the 17 biopsy specimens. C_3 deposition was noted in all cases but one. It was positive only for IgG_4.

Serum subclass distribution. The results of the distribution of circulating IgG subclasses in the serum of patients with bullous pemphigoid are summarized in Table 8. Indirect immunofluorescence examination showed IgG pemphigoid antibodies in all 25 serum samples tested. IgG_4 was present in 24 of 25 cases, IgG_1 in 14 and IgG_3 in 4 cases. IgG_4 was the only subclass detected in 11 (44%) of the total samples examined. Furthermore, in the majority of cases IgG_4 produced a more intense fluorescence than other IgG subclasses (Figure 12, A and B). Controls of skin and serum were negative for all subclasses.

Complement immunofluorescence. Complement fixing antibodies were detected in 15 (60%) out of the 25 bullous pemphigoid sera (Figure 13). To determine the relationship between complement fixing ability and the distribu-

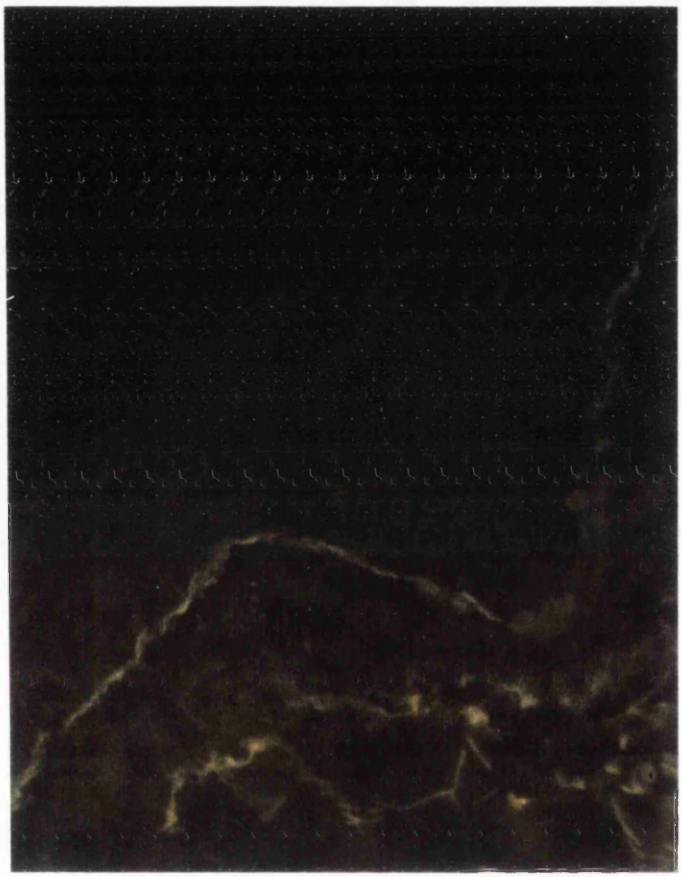


Figure 11. Direct immunofluorescence staining for IgG subclasses in the skin lesion of a patient with bullous pemphigoid.

A. Positive staining for IgG_1.

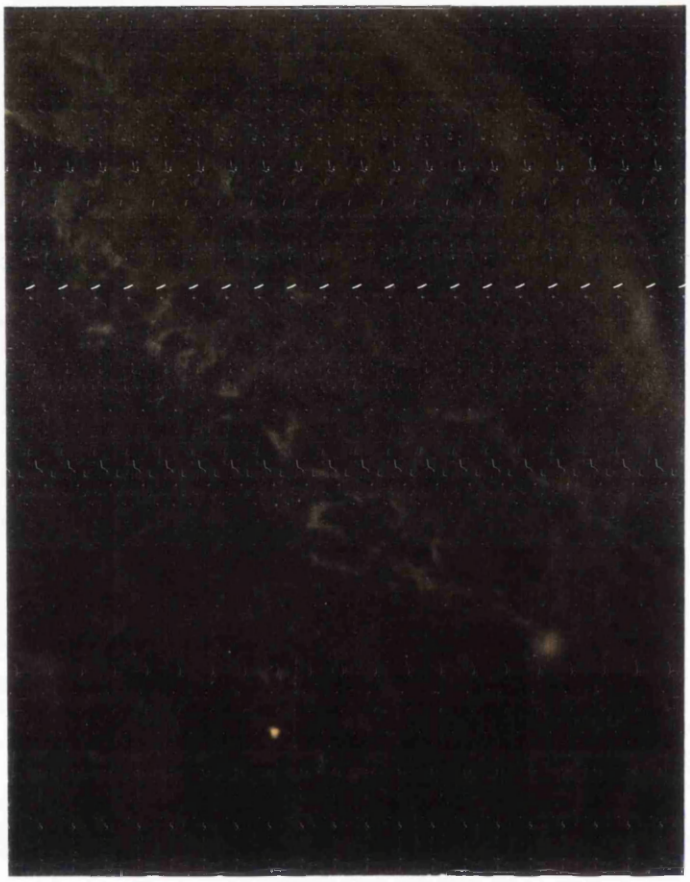


Figure 11 (continued). B. Faint staining for IgG_3.

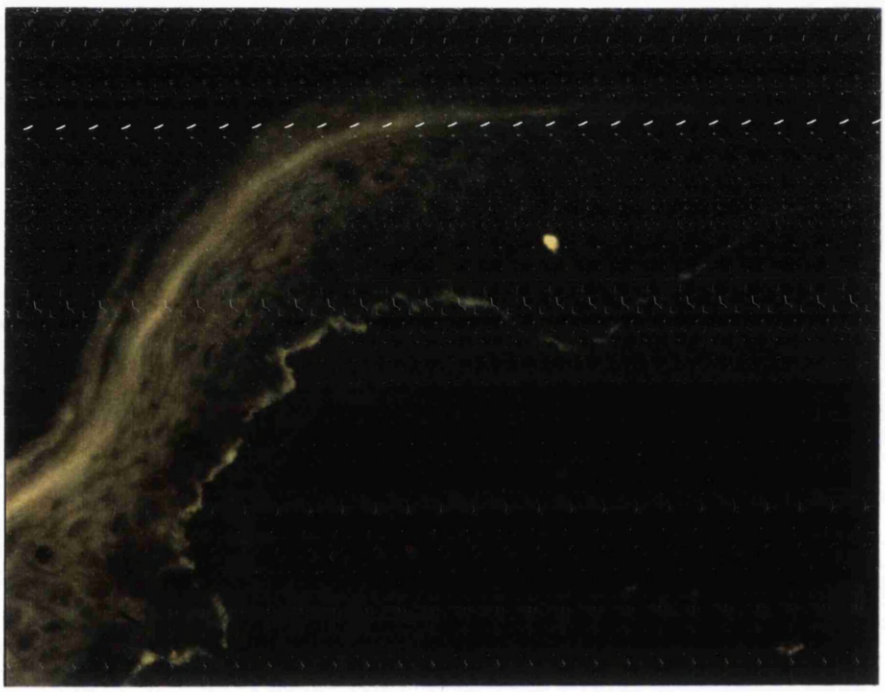


Figure 11 (continued). C. Intense staining for IgG_4.

Table 8

IgG SUBCLASSES OF CIRCULATING ANTIBODIES IN BULLOUS PEMPHIGOID

Fluorescence	IgG	IgG_1	IgG_3	IgG_4
Negative	0	11	21	1
Positive		12	4	9
Strong Positive	25	2	0	15
Total Positive	25	14	4	24
Percent Positive	100	56	16	96

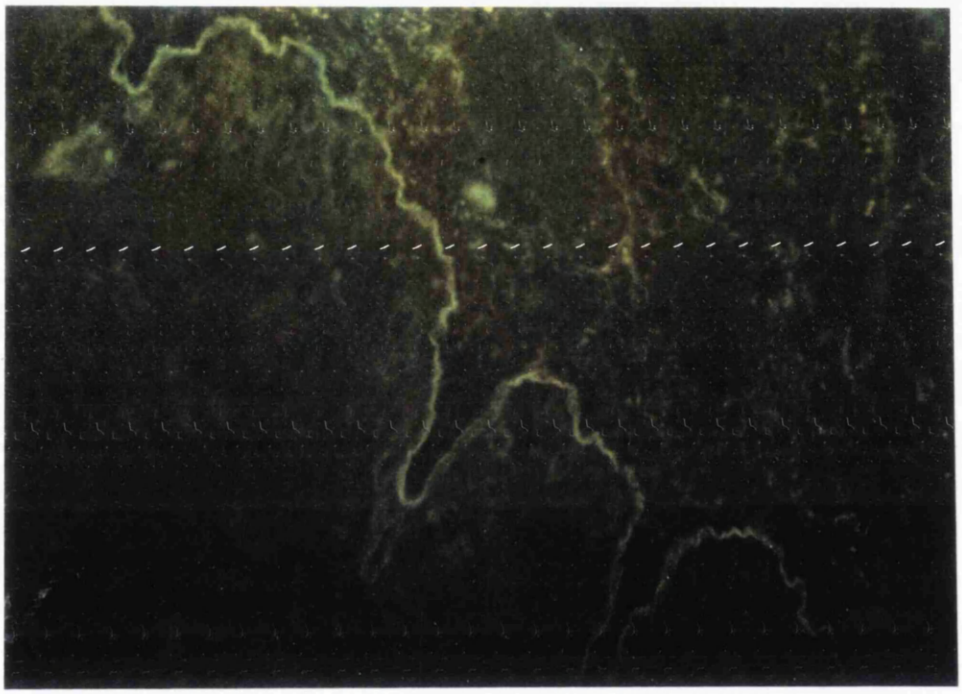


Figure 12. Indirect immunofluorescence of bullous pemphigoid patient's serum stained for IgG subclasses.

A. Positive staining for IgG_1.

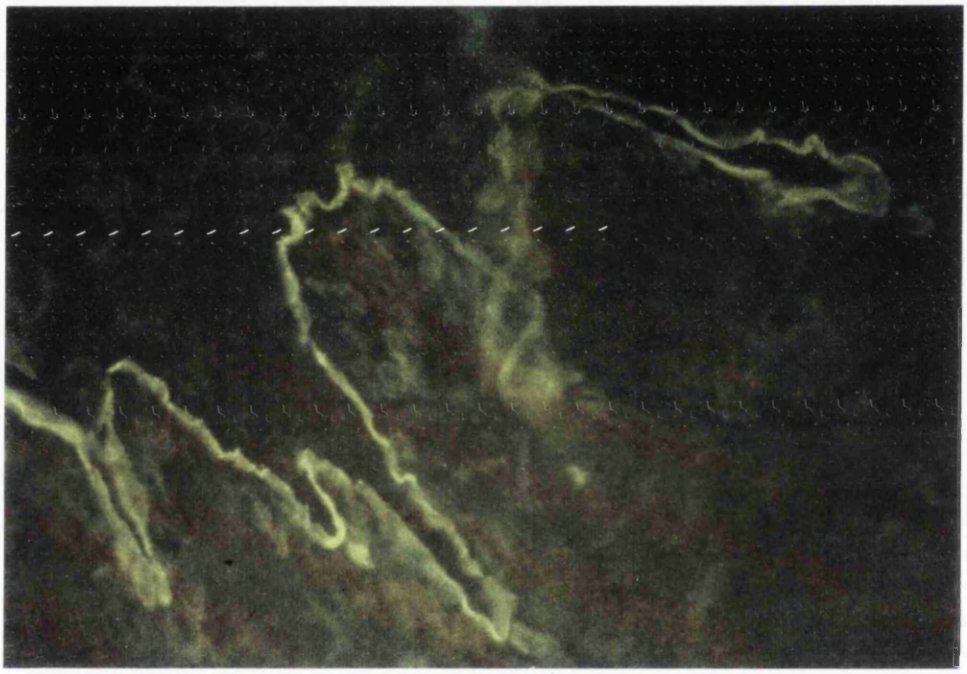


Figure 12 (continued). B. Intense staining for IgG_4.

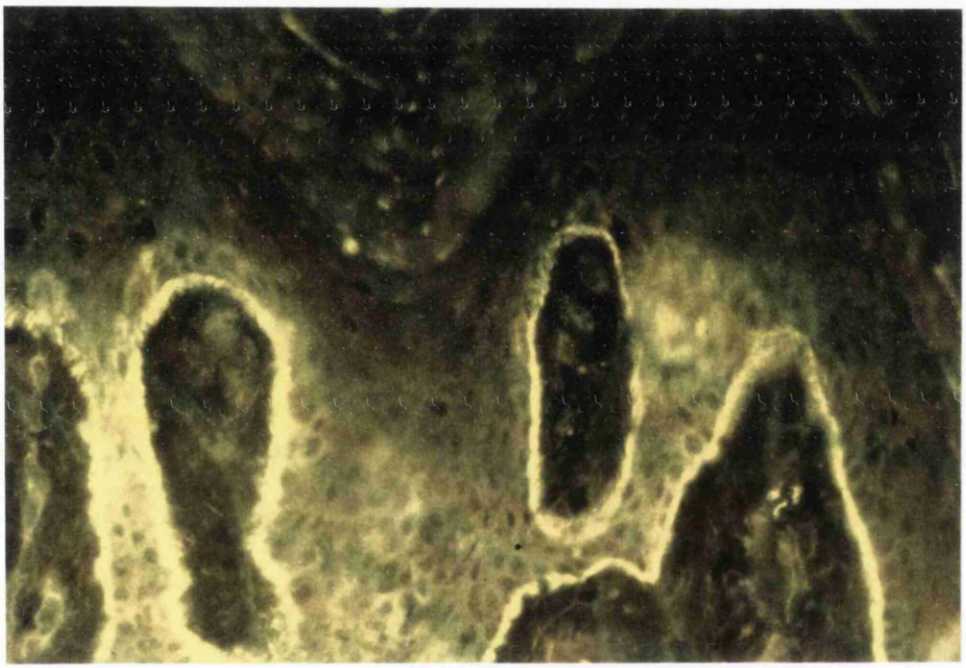


Figure 13. Positive indirect complement immunofluorescence staining of human skin with bullous pemphigoid serum.

tion of IgG subclasses, cases were grouped based on their ability to fix complement and the presence of one or both complement fixing subclasses, IgG_1 and IgG_3 (Table 9).

In 15 sera with complement fixing antibodies, 11 showed IgG_1 as well as IgG_4. In addition, 3 were positive for IgG_3. The remaining 4 samples contained only IgG_4.

In the 10 sera without complement binding antibodies IgG_4 was the only subclass observed in 7 sera; however, one or both complement fixing IgG subclasses were demonstrable in the remaining 3 samples.

3.2.2 IgG Subclasses in Pemphigus Vulgaris

Skin subclass distribution. The results of the direct immunofluorescence studies to determine the tissue distribution of IgG subclasses of intercellular antibodies are shown in Table 10. The most prominent finding on examination of skin lesions of 9 patients with pemphigus vulgaris was the demonstration of intense deposits of IgG_4 in the intercellular areas of all cases studied. Deposits of IgG_1 were detected in three patients (Figure 14, A and B). IgG_3 was not observed in any of the specimens tested. The third component of complement was present in 7 of 9 biopsies. In the two cases lacking C_3 deposits, IgG_4 was the only detectable subclass.

Table 9

RELATIONSHIP BETWEEN COMPLEMENT FIXING BASEMENT MEMBRANE ZONE ANTIBODIES AND IgG SUBCLASSES WITH COMPLEMENT ACTIVATING ABILITY

No. of Sera	IgG_1, IgG_3	IgG_4	Complement Fixing Antibodies
11	+	+	+
4	-	+	+
3	+	+	-
7	-	+	-

Table 10

IgG SUBCLASSES OF TISSUE BOUND ANTIBODIES IN PEMPHIGUS

Fluorescence	IgG	IgG_1	IgG_3	IgG_4	C_3
Negative	0	7	9	0	2
Positive	0	3	0	1	0
Strong Positive	9	0	0	8	7
Total Positive	9	3	0	9	7
Percent Positive	100	33	0	100	78

Figure 14. Direct immunofluorescence staining for IgG subclasses in the perilesional skin of a patient with pemphigus vulgaris.

A. Positive staining for IgG_1.

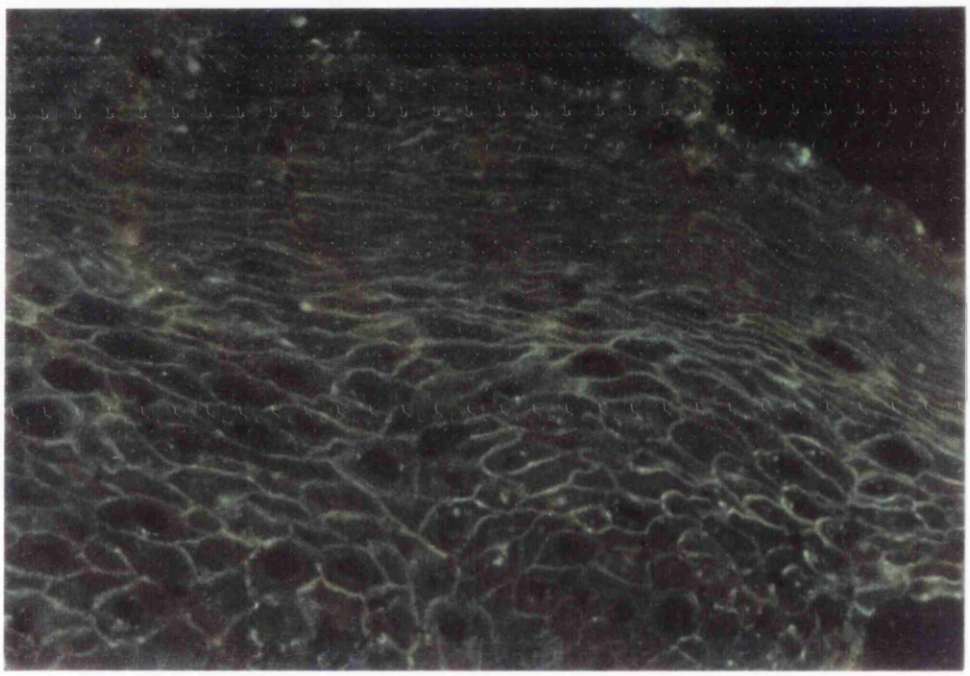


Figure 14 (continued). B. Strong intercellular staining of IgG_4.

Serum subclass distribution. The results of indirect immunofluorescence studies to determine serum subclass distribution of circulating intercellular antibodies in patients with pemphigus are shown in Table 11.

IgG subclass analysis revealed IgG_4 in all samples tested. IgG_1 was the next most frequently detected subclass, being present in 8 sera (Figure 15, A and B). IgG_4 resulted in a stronger intercellular staining in most samples. It was the only subclass found in 9 (53%) sera. IgG_3 was not detected.

Complement immunofluorescence. The relationship between the presence of complement fixing antibodies and the distribution of IgG subclasses is summarized in Table 12.

Pemphigus antibodies capable of binding complement were detected in 7 (41%) sera (Figure 16). In contrast to the bullous pemphigoid study, where the result did not show a correlation between the complement fixing antibodies and the IgG subclass distribution, the complement activating capability of intercellular antibodies appeared to be compatible with the distribution of IgG subclasses in terms of their complement fixing ability.

All the sera with complement fixing antibodies were positive for IgG_1 as well as IgG_4, whereas the non-complement fixing samples contained only IgG_4. Only one serum sample showed a negative indirect complement immunofluorescence reaction in spite of the presence of IgG_1 in addition to IgG_4.

Table 11

IgG SUBCLASSES OF CIRCULATING INTERCELLULAR ANTIBODIES IN PEMPHIGUS

Fluorescence	IgG	IgG_1	IgG_3	IgG_4
Negative	0	10	17	0
Positive	0	5	0	9
Strong Positive	17	3	0	8
Total Positive	17	8	0	17
Percent Positive	100	47	0	100

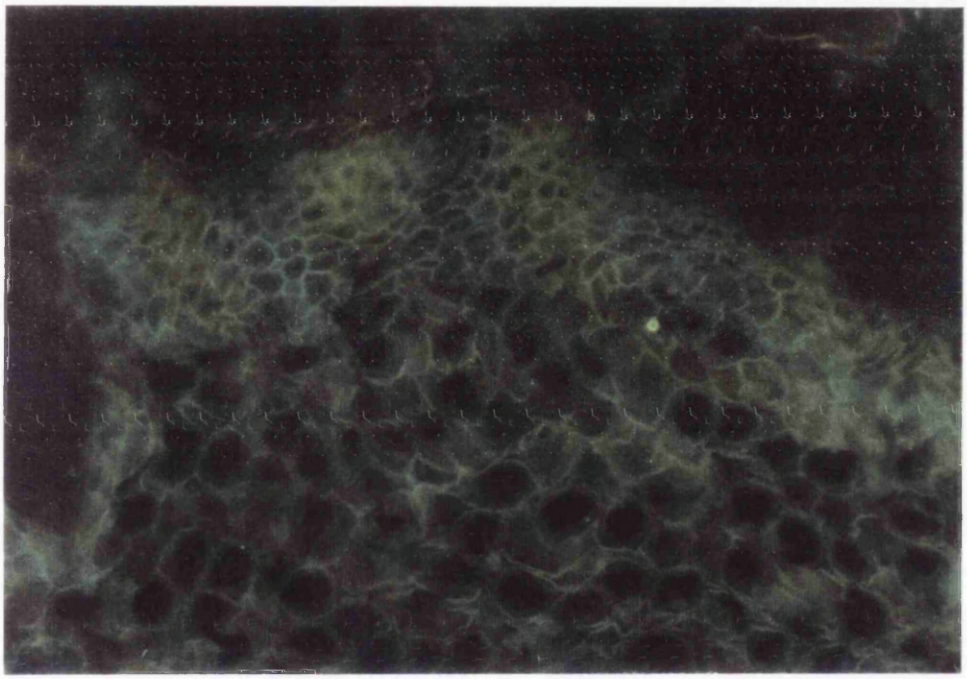


Figure 15. Indirect immunofluorescence of bullous pemphigoid patient's serum stained for IgG subclasses.

A. Positive staining for IgG_1.

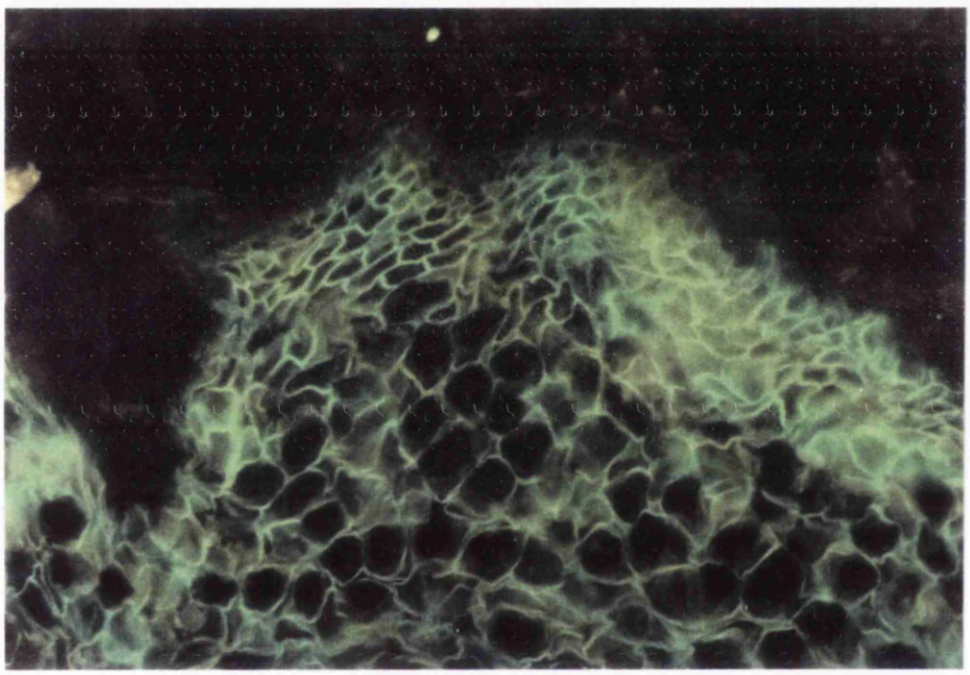


Figure 15 (continued). B. Intense intercellular staining for IgG_4.

Table 12

RELATIONSHIP BETWEEN COMPLEMENT FIXING INTERCELLULAR ANTIBODIES AND IgG SUBCLASSES WITH COMPLEMENT ACTIVATING ABILITY

No. of Sera	IgG_1	IgG_4	Complement Fixing Antibodies
7	+	+	+
9	-	+	-
1	+	+	-

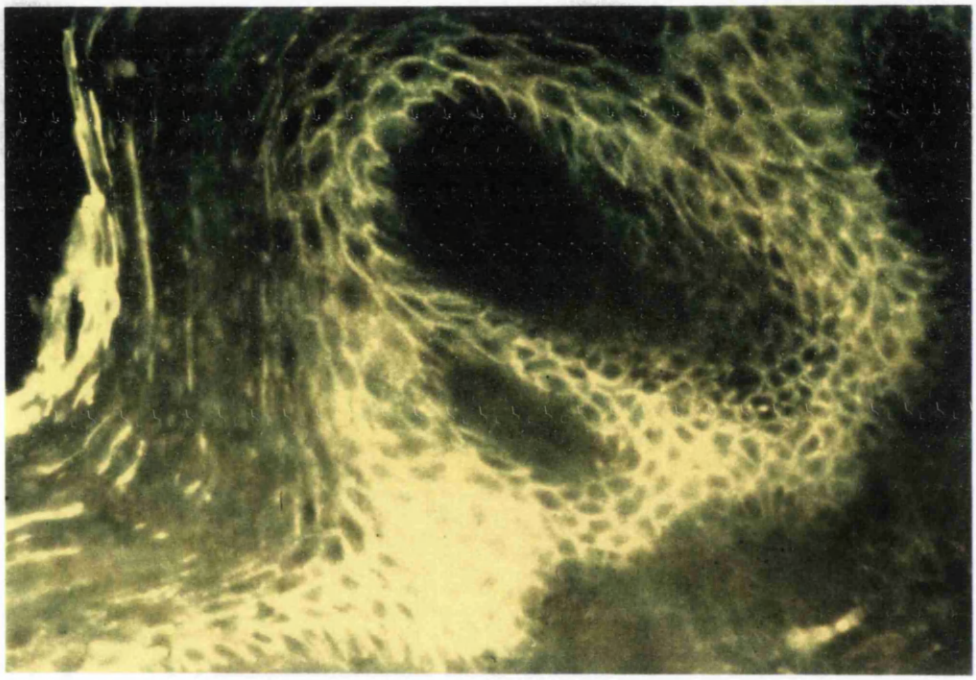


Figure 16. Positive indirect complement immunofluorescence staining of human skin with pemphigus vulgaris serum.

3.3 ANTIGEN SPECIFICITIES OF ANTI-BASEMENT MEMBRANE ZONE ANTIBODIES

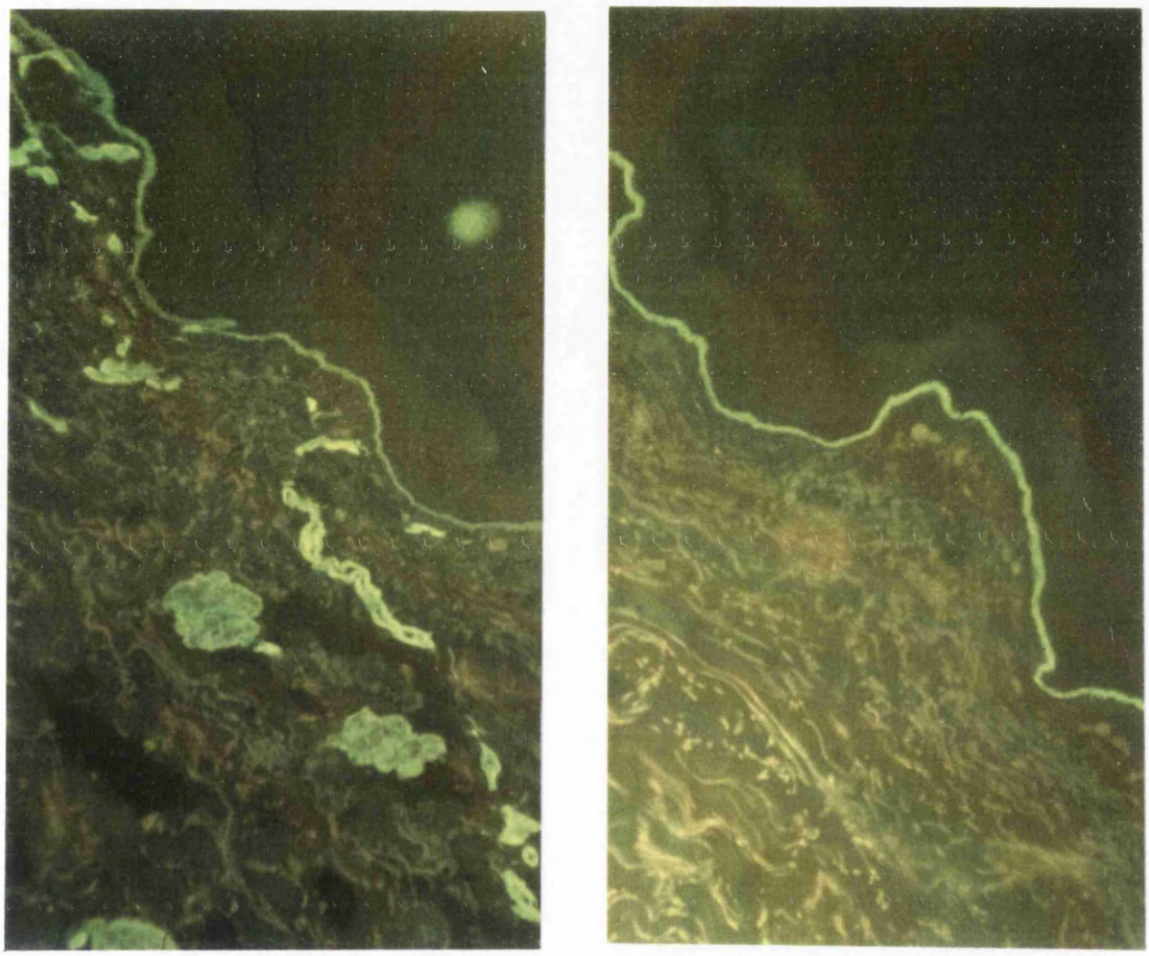
3.3.1 Localization of Type IV and Type VII Collagen on Intact and Sodium chloride-Separated Skin

Intact and frozen skin specimens of separated skin from three individuals were examined by indirect immunofluorescence using antibodies to type IV collagen and type VII collagen. Both antibodies produced a bright linear band of fluorescence at the dermal epidermal junction of intact skin (Figure 17, A and B). Anti-type IV collagen antibody also produced staining around dermal blood vessels.

On Sodium chloride-separated skin the antibody against type IV collagen resulted in a thin, sharp, continuous fluorescent band on the dermal side of the separation (Figure 18). Staining around dermal blood vessels was also noted. No staining was observed on the epidermal side.

Antibody to type VII collagen produced a broad, intense linear staining band. The fluorescence was confined to the dermal edge of the split. There was no staining of the appendages or dermal vessels (Figure 19).

These results confirm that the separation was occurring at the level of the lamina lucida.



A B

Figure 17. Linear deposition of (A) anti type IV collagen antibody and (B) with anti type VII collagen antibody at the basement membrane zone of normal intact human skin.

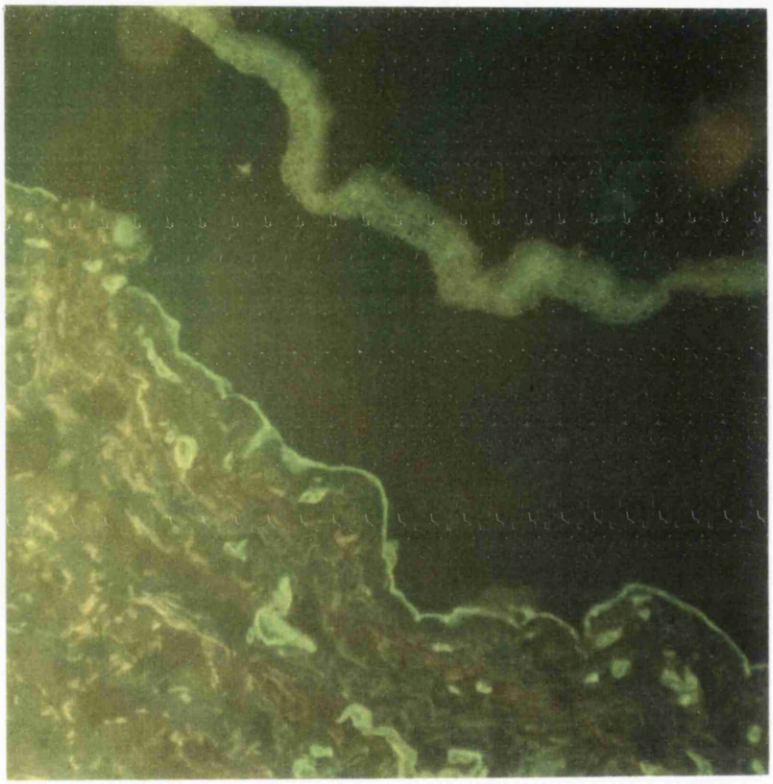


Figure 18. Sodium chloride-separated skin showing fluorescence on the dermal side of the split with anti type IV collagen antibody.

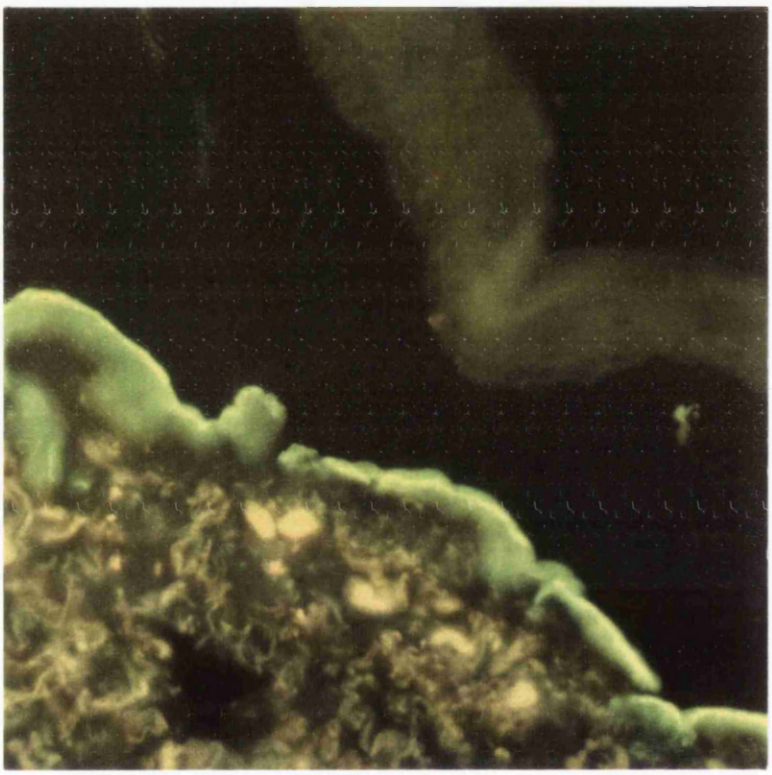


Figure 19. Sodium chloride-separated skin showing fluorescence on the dermal side of the split with anti type VII collagen antibody.

3.3.2 Indirect Immunofluorescence of Bullous Pemphigoid and Epidermolysis Bullosa Acquisita Antibodies on Split-Skin

Each of the test sera was examined at a titre of 1:10 on cryostat sections of separated skin obtained from one specimen. Two patterns of binding were noted. Staining was either of the epidermis (roof of split) or dermis (floor of split). A mixed (combined) staining pattern of the epidermis and dermis has not been seen in any of the cases tested.

All 27 bullous pemphigoid sera demonstrated an epidermal staining pattern (Figure 20). None of the sera produced a dermal pattern.

The epidermolysis bullosa acquisita serum produced fluorescence along the dermal side of separation (Figure 21). All control sera were negative on split-skin.

To determine the reproducibility of the staining patterns, the epidermolysis bullosa acquisita and 12 bullous pemphigoid sera were tested on separated skin sections obtained from two other specimens. The staining pattern was identical on all specimens.

To study the sensitivity of sodium chloride-separated skin as a substrate for detecting anti-basement membrane zone antibodies, the epidermolysis bullosa acquisita and 8 bullous pemphigoid sera were titrated in doubling dilutions and simultaneously tested on intact and separated skin. The results are shown in Table 13. Two of the bu-

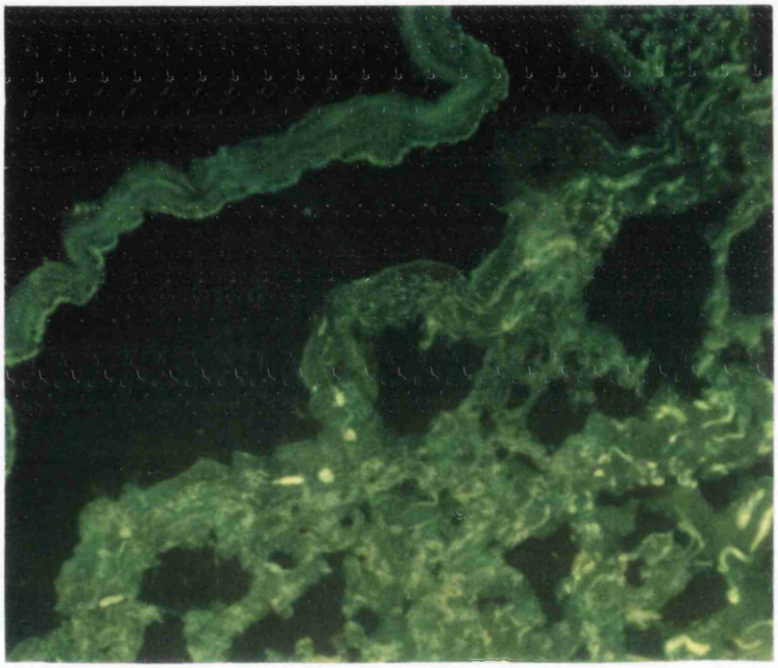


Figure 20. Sodium chloride-separated skin with bullous pemphigoid serum, showing linear fluorescence on the epidermal side.

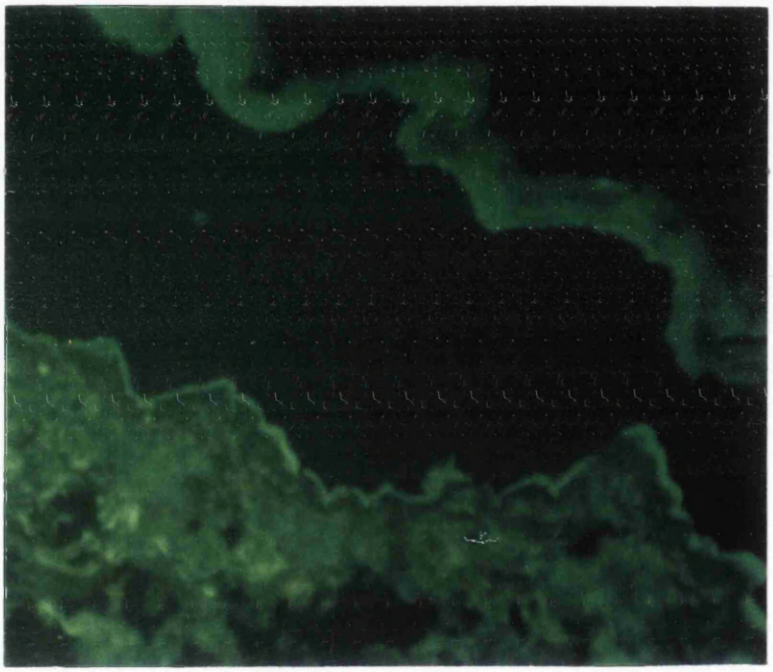


Figure 21. Sodium chloride-separated skin with epidermolysis bullosa acquisita serum, showing dermal pattern of fluorescence.

Table 13

INDIRECT IMMUNOFLUORESCENCE USING
BULLOUS PEMPHIGOID AND EPIDERMOLYSIS BULLOSA ACQUISITA
SERA ON INTACT AND SPLIT SKIN

Disease	Serum No.	Intact Skin	Split Skin
Bullous pemphigoid	1	1:80	1:160
	2	1:320	1:640
	3	1:40	1:160
	4	1:640	1:640
	5	1:160	1:320
	6	1:160	1:320
	7	1:160	1:320
	8	1:160	1:160
Epidermolysis bullosa acquisita	1	1:160	1:320

llous pemphigoid sera demonstrated equal titres on both substrates while the epidermolysis bullosa acquisita serum and the remainder of the bullous pemphigoid sera showed titres on split skin that were 1-2 dilutions greater on split skin than on intact skin.

3.3.3 Immunofluorescence Studies of Sodium Chloride-Separated Biopsy Specimens

In addition to performing IgG and C_3 direct immunofluorescence studies to determine the pattern of fluorescence on sodium chloride-separated specimens, sections from each biopsy were simultaneously reacted with monoclonal antibodies against type IV collagen and type VII collagen. The antibodies produced staining along the dermal edge of the split in all cases (Figures 22 and 23). This finding indicated that the ultrastructural level of separation was occurring at the level of the lamina lucida. Separation beneath the lamina densa would have resulted in an epidermal pattern of fluorescence.

Table 14 shows the result of testing Sodium Chloride-separated biopsy specimens from seven patients with bullous pemphigoid. Attempts to separate a specimen from an epidermolysis bullosa acquisita patient were unsuccessful. Direct IgG and C_3 immunofluorescence showed staining on the epidermal side of the separation in six patients

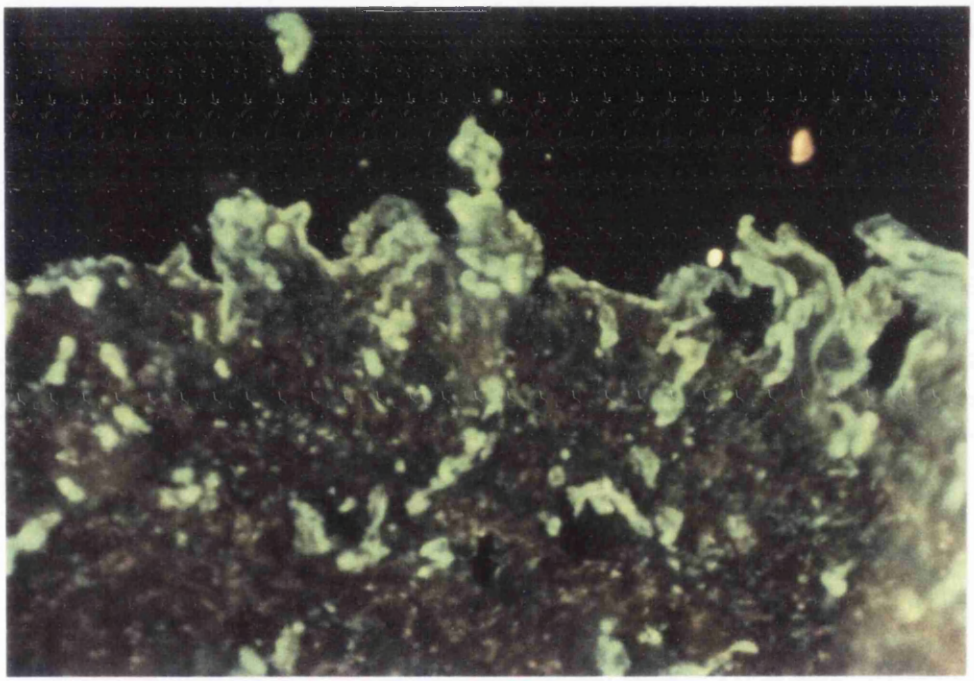


Figure 22. Sodium chloride-separated pemphigoid biopsy specimen staining the dermal side of the split with anti type IV antibody.

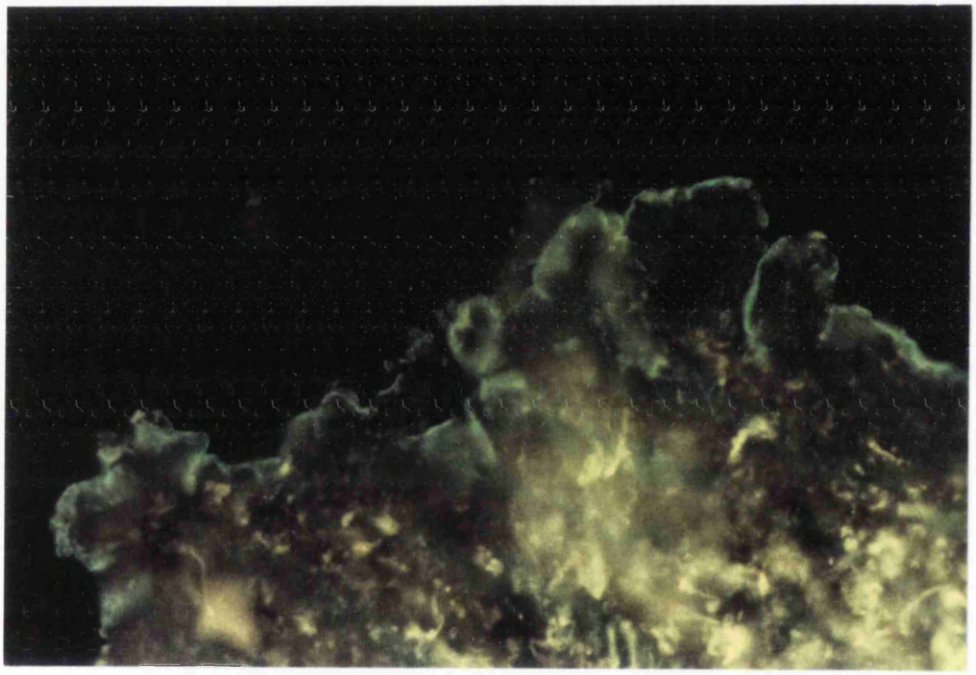


Figure 23. Sodium chloride-separated bullous pemphigoid biopsy specimen staining the dermal side of the split with anti type VII antibody.

Table 14

DIRECT IMMUNOFLUORESCENCE OF
BULLOUS PEMPHIGOID BIOPSIES SEPARATED BY SODIUM CHLORIDE

Patient No.	Antibody to	Fluorescence Pattern		
		E	C	D
1	IgG	+		
	C_3	+		
2	IgG	+		
	C_3	+		
3	IgG		+	
	C_3		+	
4	IgG	+		
	C_3	+		
5	IgG	+		
	C_3	+		
6	IgG	+		
	C_3	+		
7	IgG	+		
	C_3	+		

E = epidermal
C = combined, epidermal and dermal
D = dermal

(Figure 24). In one patient the deposits were observed on both the epidermal and dermal sides of the split (Figure 25).

3.3.4 SDS-PAGE and Western Immunoblotting

Ponceau S staining. Staining with this reagent was performed on blots to visualize protein bands and thus confirm that electrophoretic transfer of proteins has taken place. Figure 26 shows a nitrocellulose sheet stained with Ponceau S and indicates the total protein transferred in each lane in addition to the simultaneously run molecular weight standards.

Protein content estimation. Lowry's assay was used to determine the protein content in epidermal and dermal extracts. The total protein in the epidermal extract was approximately 3.75mg/ml and the dermal extract contained 3.0mg/ml.

Western immunoblotting patterns. The skin antigens defined by one epidermolysis bullosa acquisita and seven bullous pemphigoid serum samples were investigated by Western immunoblotting. The pattern of protein bands demonstrated by different sera is shown in Table 15. Five of the seven samples tested had antibodies to specific antigens that could be visualized in immunoblots. Four of the five positive sera recognized a protein with a

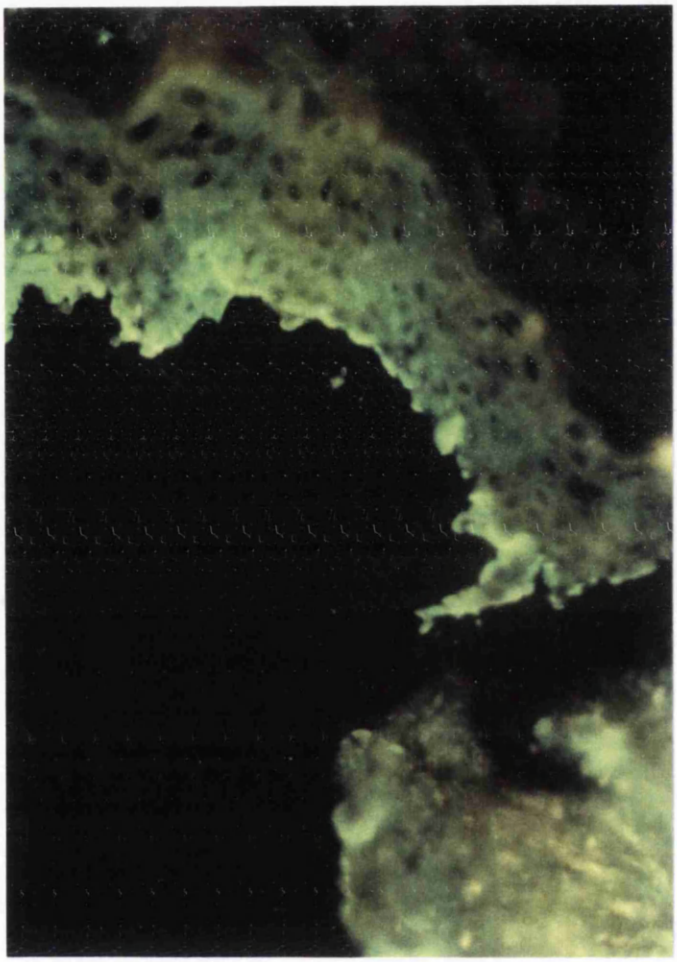


Figure 24. Direct immunofluorescence of a bullous pemphigoid biopsy specimen separated through the lamina lucida. Linear deposits of IgG are present along the epidermal side of the split.

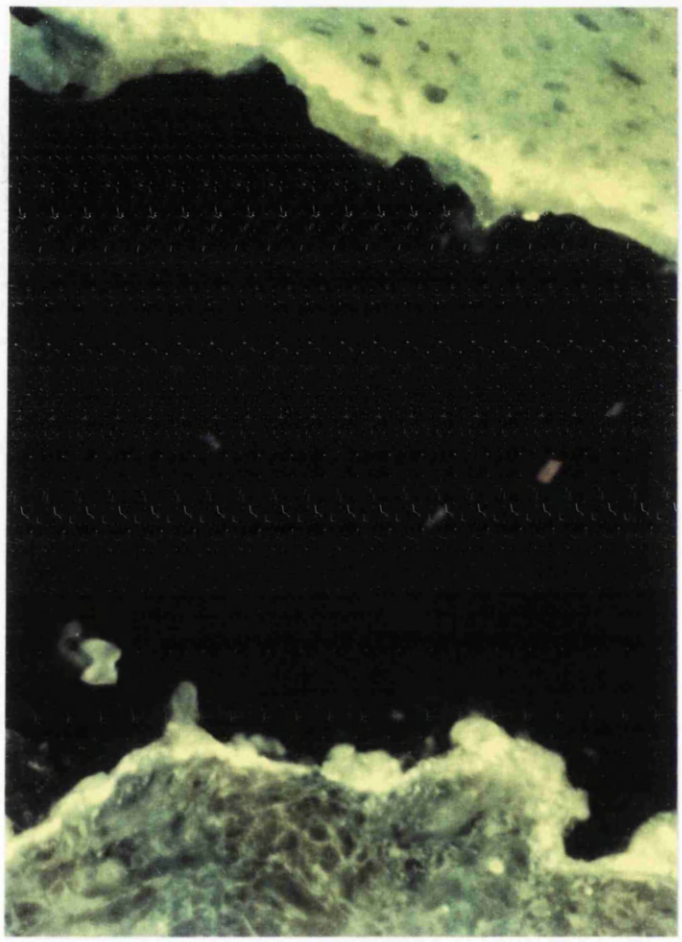


Figure 25. Direct immunofluorescence of separated bullous pemphigoid biopsy specimen showing an epidermal-dermal staining pattern.

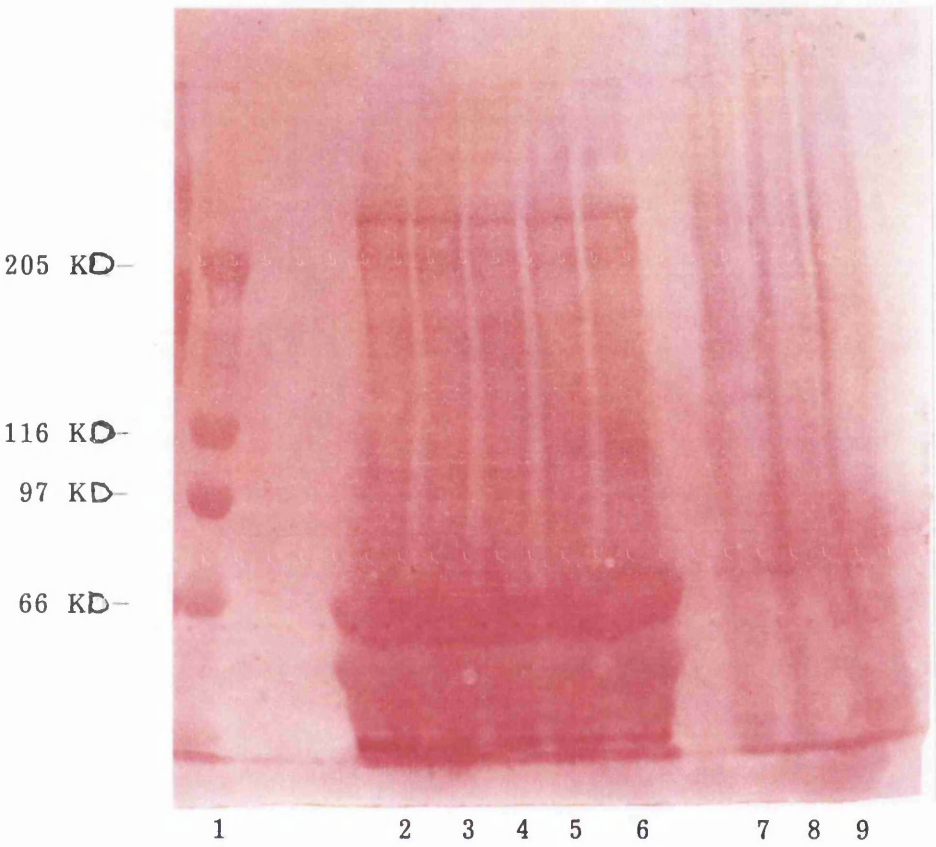


Figure 26. A nitrocellulose sheet stained with Ponceau S. Lane 1 shows the molecular weight markers. Lanes 2-5 and 7-9 show the epidermal and dermal extracts respectively.

Table 15

ANTIGENS RECOGNIZED BY
ANTI-BASEMENT MEMBRANE ZONE ANTIBODIES
IN WESTERN IMMUNOBLOTTING

Disease	Serum No.	Titre	Molecular Weight (KD)
Bullous pemphigoid	1	1:160	-
	2	1:320	-
	3	1:160	220
	4	1:640	220
	5	1:320	220
	6	1:160	180
	7	1:640	220
Epidermolysis bullosa acquisita	1	1:640	-

molecular weight of approximately 220 Kd. The remaining positive sample reacted to a 180 Kd protein and a faint band was also observed at 205 Kd. Figure 27 shows examples of two pemphigoid and two control sera. The proteins recognized in this assay were extracted from the epidermis. They were not detected in the dermal extract. Two bullous pemphigoid sera and the epidermolysis bullosa acquisita serum did not react with any protein in either the dermal or epidermal extracts. Bands staining below 66 Kd were considered non specific because they were seen with some bullous pemphigoid as well as control sera. All controls did not detect any major antigen in either epidermal or dermal extracts.

3.4 ENDOMYSIAL ANTIBODIES IN BULLOUS DISEASES

The diagnosis of dermatitis herpetiformis was confirmed by the demonstration of granular papillary IgA deposits in normal appearing skin (Figure 28).

The specific indirect immunofluorescence pattern of the IgA anti-endomysial antibodies on the smooth muscle of monkey oesophagus is illustrated in Figures 29 and 30. The staining reaction appears as a network of thin lines in the connective tissue surrounding individual muscle fibres. Figure 31 shows a negative reaction for en-

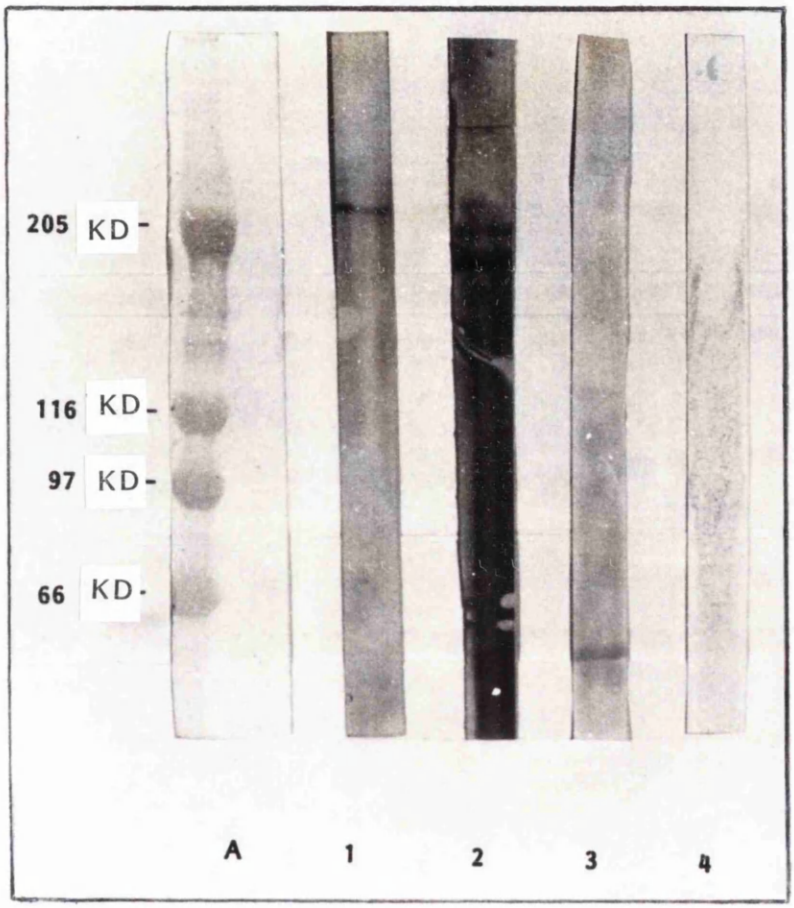


Figure 27. Immunoblot of epidermal extract with bullous pemphigoid sera reacting with 220 KD on lane 1 or a 180 KD on lane 2. A non specific reaction is seen with normal human serum on lane 3. A negative reaction on lane 4 with serum from a pemphigus patient. Concurrently electrophoresed standard proteins are in lane A.

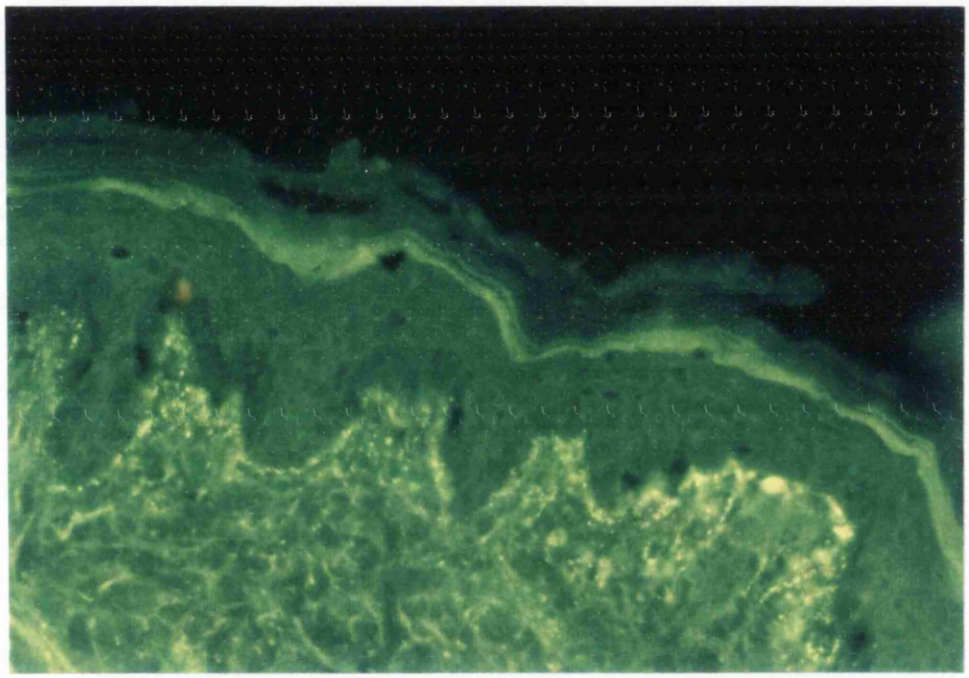


Figure 28. Direct immunofluorescence of dermatitis herpetiformis showing granular IgA deposits at the dermal papillae.

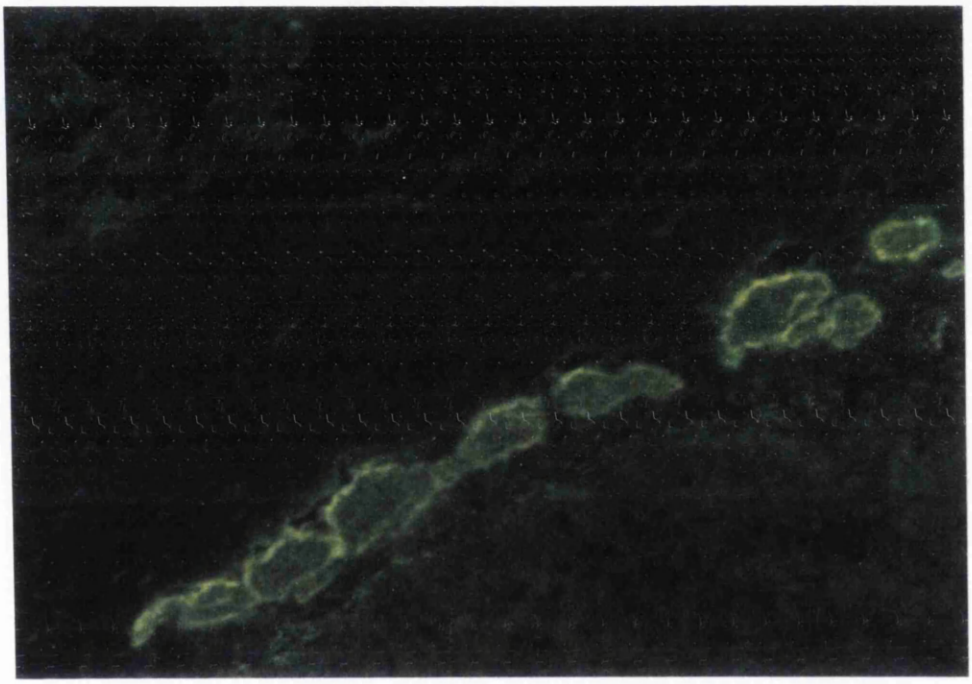


Figure 29. Low magnification of monkey oesophagus section treated with a 1:2.5 dilution of a positive serum for endomysium antibodies.

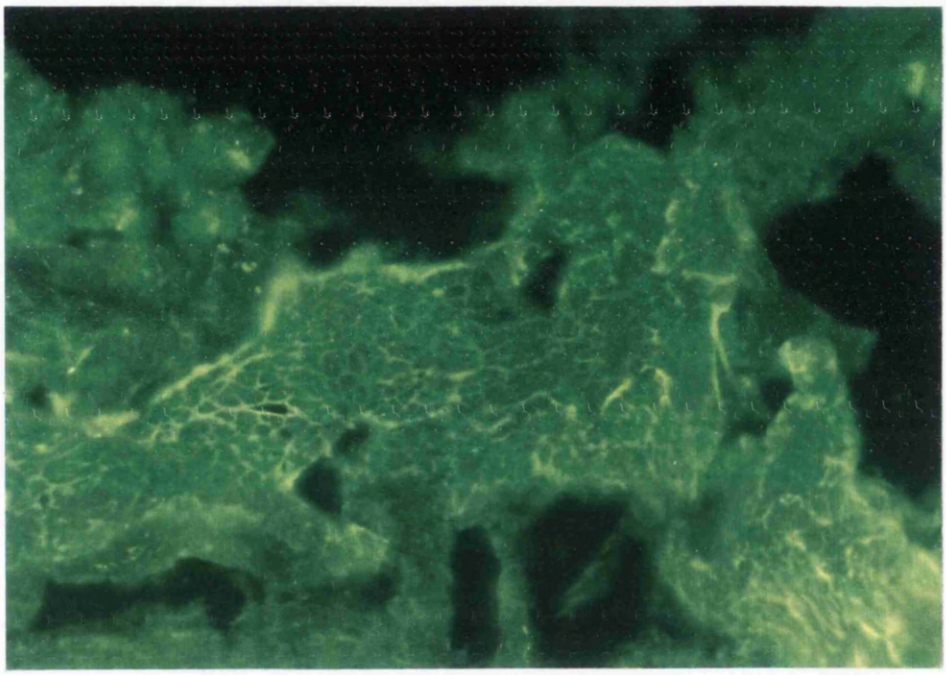


Figure 30. Higher magnification of oesophagus section showing reticulin-like pattern in the smooth muscle layer adjacent to epithelium.

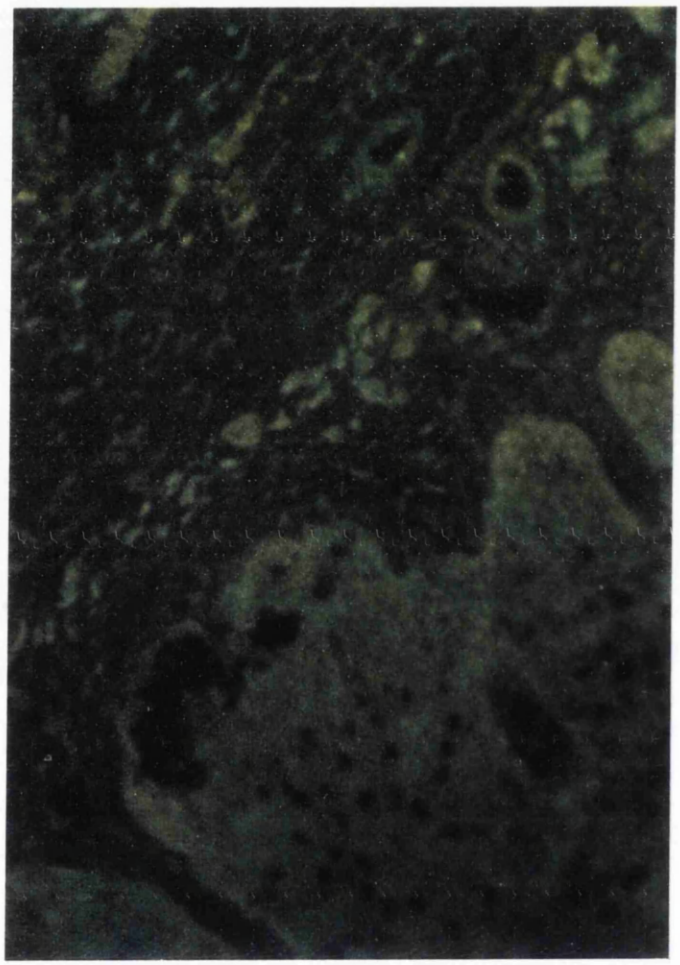


Figure 31. Monkey oesophagus section treated with pemphigus serum and FITC conjugated IgA. Note negative reaction.

domysial antibodies in a 1:2.5 dilution of a control serum from a patient with pemphigus. In contrast to endomysial antibodies, anti-smooth muscle antibodies which are commonly found at low titres react only to the sarcoplasm producing a homogenous fluorescence of myofibrils (Figure 32).

The immunofluorescence findings obtained with sera from dermatitis herpetiformis patients and control sera from patients with bullous pemphigoid and pemphigus are summarized in Table 16.

Circulating endomysium antibodies were detected in 60% of the dermatitis herpetiformis cases in titres that ranged from 1:2.5 to 1:160 (Table 17). However, a higher frequency of the antibodies was observed in patients on a normal, gluten-containing diet. The endomysium antibodies were demonstrated in 11 (73%) of 15 patients who were on a normal diet. Conversely, the antibodies occurred in only one (20%) of the five patients adhering to a gluten-free diet.

The endomysium antibodies could not be detected in any of the 40 control sera from bullous pemphigoid and pemphigus patients.

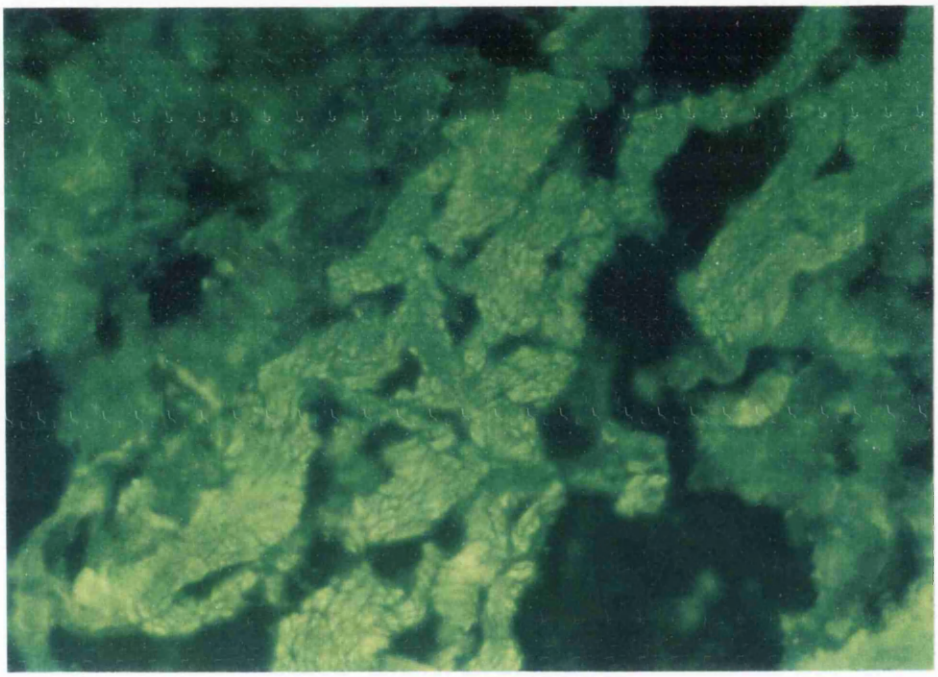


Figure 32. Indirect immunofluorescence of anti-smooth muscle antibodies of the IgA class. Note the homogenous fluorescence of the smooth muscle sarcoplasm.

Table 16

FREQUENCY OF IgA CLASS ANTI-ENDOMYSIAL ANTIBODIES IN DERMATITIS HERPETIFORMIS, BULLOUS PEMPHIGOID AND PEMPHIGUS

	No. of Samples Tested	No. with Positive Titre	Percent
Dermatitis herpetiformis (total)	20	12	60
Dermatitis herpetiformis (normal diet)	15	11	73
Dermatitis herpetiformis (gluten-free diet)	5	1	20
Bullous pemphigoid	20	0	0
Pemphigus	20	0	0

Table 17

ANTI-ENDOMYSIAL ANTIBODY TITRES
IN DERMATITIS HERPETIFORMIS PATIENTS
ON NORMAL OR GLUTEN-FREE DIET

	Anti-Endomysial Antibody Titre							
Diet	Neg.	2.5	5	10	20	40	80	160
Normal	4	1	1	2	4	1	1	1
Gluten-free	4		1					

4. DISCUSSION

4.1 SELECTION OF EPITHELIAL TISSUE FOR INDIRECT IMMUNOFLUORESCENCE STAINING OF BULLOUS PEMPHIGOID AND PEMPHIGUS ANTIBODIES

Indirect immunofluorescence is used to detect circulating anti-basement membrane zone and intercellular antibodies (referred to as antiepithelial antibodies) which can be identified by their distinct staining patterns on various antigenic substrates.

Failure to demonstrate the serum autoantibodies in some patients with bullous pemphigoid or pemphigus vulgaris is not the result of species specificity of circulating antibodies. Species specificity studies have shown that basement membrane and intercellular antibodies are not species specific and react with human skin and tissue from different mammals[46]. However, the intercellular antibodies of pemphigus are organ specific and react only with squamous epithelium[46]. Normal human skin, which contains both bullous pemphigoid and pemphigus antigens, should be the ideal substrate to test for serum pemphigoid and pemphigus antibodies. However, it remains controver-

sial which substrate is superior for the demonstration of these autoantibodies because of conflicting data obtained by different investigators.

Although normal human skin is the natural substrate for the antiepithelial antibodies to react with, several studies have suggested that primate tissue is the ideal substrate for the detection of these antibodies[46,173]. In addition, other reports indicated that human skin is not a sensitive enough substrate to use in indirect immunofluorescence assays[61,62,179]. However, this conclusion may be inaccurate in light of the reported regional[4,180] and individual[181] variations in the expression of bullous pemphigoid and pemphigus vulgaris antigens.

In the present study the expression of both bullous pemphigoid and pemphigus vulgaris antigens was investigated. Subsequently, sera from patients with these conditions were simultaneously assayed on standard animal substrates in addition to normal human skin with strong antigen expression. The findings of this investigation suggest that the bullous pemphigoid antigen is not uniformly expressed over the body but rather shows variations between different sites. The expression of this antigen estimated by reacting sera containing bullous pemphigoid antibodies with specimens of normal skin from face, trunk and flexural lower limb showed a high concentration of bullous pemphigoid antigen in the face.

Skin from the limb also showed strong antigen expression. However, much lesser amounts were present in the skin obtained from the trunk. In contrast to bullous pemphigoid antigen, the degree of variation in the expression of pemphigus vulgaris antigen was minimal. The results of this study showed that the antigen was more uniformly distributed over the body. The expression of antigen was similar in the areas of the face, trunk and limb. When bullous pemphigoid and pemphigus vulgaris antigen distribution studies were repeated it became evident that the expression of an antigen was similar in skin specimens obtained from the same region in different individuals but may differ in skin obtained from different sites. These observations indicate that variations in antigen expression cannot be attributed entirely to differences in antigen concentration between individuals. In contrast to the findings of a previous investigation[4], the results of antigen expression in this study did not correlate well with the distribution of lesions in bullous pemphigoid. Although the incidence of facial blisters is low in pemphigoid, high concentrations of antigen were found in skin biopsy specimens obtained from that area. This discrepancy in the results may be due to variations in the specificity of anti-basement membrane zone antibodies in different sera. In addition, it must be stressed that little is known about the different factors involved in determining the distribution of lesions in various blis-

tering disorders. Thus the involvement of factors other than antigen expression in the localization of lesions in bullous pemphigoid and pemphigus vulgaris cannot be ruled out.

Choice of substrate.

The variability in the expression of bullous pemphigoid and pemphigus vulgaris antigens must be considered when normal human skin is used as a substrate in indirect immunofluorescence assays.

The diagnostic significance of immunofluorescence tests for antiepithelial antibodies of bullous pemphigoid and pemphigus underlines the importance of selecting a highly reactive substrate for serum antibody determinations in these disorders. In this study the sensitivity of normal human skin was compared with two other epithelial substrates commonly used in indirect immunofluorescence testing. The results showed that the titres of circulating antibodies may vary depending on the type of epithelial substrate used in the immunofluorescence assay. The investigation involved testing for circulating antiepithelial antibodies in the sera of 21 patients with bullous pemphigoid and 15 patients with pemphigus vulgaris against three antigenic substrates - normal skin from the face, monkey oesophagus and guinea pig lip. The sensitivity of the substrates was evaluated by the concurrent testing of each serum sample on all three epithelial sources. The most sensitive substrate for the

detection of both bullous pemphigoid and pemphigus vulgaris antibodies was monkey oesophagus. The highest titres were most frequently observed when this substrate was used. This finding is in accordance with previous reports[173,182]. The study also demonstrated the usefulness and reliability of guinea pig lip for serum antibody estimation in bullous pemphigoid and pemphigus vulgaris. Many of the serum samples examined produced equal titres on guinea pig lip and monkey oesophagus. This is in contrast to the findings in other studies[73,183] which suggested that guinea pig lip is inferior to other animal substrates and normal human skin for the detection and titration of bullous pemphigoid and pemphigus antibodies.

Although it has been suggested that normal skin, obtained from an appropriate site, is as sensitive or even superior to monkey oesophagus for the detection of antiepithelial antibodies[184], the findings of the present study showed normal skin to yield lower basement membrane zone and intercellular antibody titres compared to monkey oesophagus despite using tissue from an area rich in both bullous pemphigoid and pemphigus vulgaris antigens. However, the titre difference was mostly confined to one or two doubling dilutions. A difference of three dilutions was only observed in one case of bullous pemphigoid and two cases of pemphigus vulgaris.

Site differences.

Differences in the expression of bullous pemphigoid and pemphigus antigens have been described in the literature. Table 18 indicates areas with the greatest antigen expression reported in different studies.

Bullous pemphigoid. Using three bullous pemphigoid sera, Goldberg et al.[4] determined the end point titre variations in 46 different specimens of normal human skin and found that there were marked differences in the concentration of bullous pemphigoid antigen at various sites. They observed that the highest amounts of antigen were in skin obtained from the flexor surfaces; flexor arm, flexor thigh and popliteal fossa. The lowest concentration of bullous pemphigoid antigen was in the scalp, face and extensor surfaces of the arm. Skin from trunk, knee and extensors of thighs had intermediate amounts of antigen. The expression of bullous pemphigoid antigen from the same site in different individuals was comparable. Interestingly, the highest concentration of antigen was in areas commonly involved in the disease (flexural surfaces), whereas the lowest was in areas (head and extensor arm) which are rarely involved. This apparent correlation between the expression of antigen and the location of lesions in bullous pemphigoid suggests that the concentration of bullous pemphigoid antigen may play a

Table 18

BODY SITES WITH THE GREATEST EXPRESSION OF BULLOUS PEMPHIGOID AND PEMPHIGUS VULGARIS ANTIGENS

Date	Study Group	Bullous Pemphigoid	Pemphigus Vulgaris
1984	Goldberg et al.(4)	Flexor arm, flexor thigh and popliteal fossa	-
1986	Sison Fonacier and Bystryn(180)	-	Axilla, scalp, face, buccal mucosa
1988	Hamm and Wozniak(185)	Plantar sites, sacral region	-
1991	Ioannides et al.(186)	-	Buccal mucosa, scalp, face
1992	Alkarawi	Face and limb	Face, trunk, limb

role in the distribution of lesions in this disease[4]. Hamn and Wozniak[185] also reported regional variations in the expression of bullous pemphigoid antigen in normal skin, as estimated by end point titres of antibody in sera of two patients with bullous pemphigoid. The study confirmed that the amount of bullous pemphigoid antigen varies markedly at different sites on the body. They found that the highest antibody titres were obtained in assays using skin from the plantar region, whereas the lowest titres were observed when skin from flexural areas was used as a substrate. In addition, bullous pemphigoid antigen expression in relation to age was also investigated. Studies of skin samples from cadavers of different age groups showed that the highest end point titres were found in the oldest individuals examined and the lowest in the youngest.

Differences in antibody titres of bullous pemphigoid sera on different biopsy specimens were first described by Zhu and Bystryn[181]. They studied 16 serum samples against five skin specimens from different individuals. Their observation suggested that the expression of bullous pemphigoid antigen is not an all or none phenomenon but that it differs between individuals.

Pemphigus. Studies have also been carried out to determine the concentration of pemphigus vulgaris antigen in various body regions. Sison Fonacier and Bystryn[180] studied three sera with pemphigus vulgaris antibodies

against 46 skin specimens obtained from different individuals. They noted variations in pemphigus vulgaris antigen expression depending on the site from which the biopsy specimen was obtained. They found that antigen concentration was greatest on scalp, face, axilla, buccal mucosa and neck. It was lowest on skin from lower back and groin. There was an intermediate expression of pemphigus vulgaris antigen in other areas: chest, abdomen, upper back and legs. Similar observations were made by Ionnides et al.[186]. They found that the greatest expression of antigen determined from the highest antibody titres of two pemphigus vulgaris sera was in the buccal mucosa, scalp and face. These studies, therefore, demonstrate that the pemphigus vulgaris antigen is strongly expressed in sites frequently affected by pemphigus lesions whereas antigen expression is poor in areas that are usually spared.

Substrates.

Differences in the reactivity of antiepithelial antibodies on various substrates have also been reported. Discrepancy in the results obtained in different studies led to controversy regarding the most suitable tissue for the detection and titration of these antibodies.

Bullous pemphigoid. Table 19 indicates the substrates used in different investigations. Beutner et al.[45] studied the use of guinea pig lip and human skin for the demonstration of basement membrane zone antibodies in

Table 19

EPITHELIAL SUBSTRATES USED FOR THE DETECTION OF BULLOUS PEMPHIGOID ANTIBODIES

Date	Study Group	Substrates Tested	Recommended Substrate
1967	Beutner et al.(45)	Guinea pig lip, human skin	Guinea pig lip
1969	Wick and Beutner(187)	Guinea pig and monkey lip	Guinea pig lip
1969	Katz et al.(64)	Normal human skin and rabbit oesophagus	Human skin
1972	Chorzelski and Beutner(61)	Monkey, rabbit and guinea pig lip	Monkey lip mucosa
1977	Gligora et al.(189)	Human tonsil and monkey oesophagus	Human tonsil
1985	Goldberg et al.(184)	Normal skin, monkey and guinea pig oesophagus	Normal skin (flexural site)
1986	Bystryn and Sabolinski(62)	Normal skin, monkey and guinea pig oesophagus	Guinea pig oesophagus
1992	Alkarawi	Normal skin, monkey oesophagus and guinea pig lip	Monkey oesophagus

bullous pemphigoid. They considered guinea pig lip more sensitive than human skin. The authors suggested that the low titres obtained on human skin sections were due to immunoglobulin staining of the dermis which obscured basement membrane zone fluorescence. Conversely, such dermal staining was not observed on guinea pig lip sections. In another report Wick and Beutner[187] studied two bullous pemphigoid sera using monkey and guinea pig lip and mucosa. They confirmed the value of guinea pig lip as a substrate for the detection of bullous pemphigoid antibodies. In contrast, other studies have suggested that tissue of primate origin is the most reliable for the demonstration of these antibodies[46,173]. Chorzelski et al.[61] evaluated the reactivity of three bullous pemphigoid sera by indirect immunofluorescence on monkey, rabbit and guinea pig lip tissue. They found that only one serum showed a positive reaction on all three substrates whereas the other two sera reacted with monkey tissue but failed to react with any of the rabbit and guinea pig tissue. Other authors have observed that human skin is a reliable substrate in indirect immunofluorescence assays. Muller et al.[188] found that normal human skin was as sensitive as guinea pig oesophagus for antibody estimation in bullous pemphigoid. Katz et al.[64] compared the affinity of human skin and rabbit oesophagus for anti-basement membrane zone antibodies and found that two bullous pemphigoid sera yielded a positive reaction only when human skin was used

as a substrate. In a study comparing human skin with monkey and guinea pig oesophagus, bullous pemphigoid antibodies were detected on human skin in 10% of sera that failed to react with animal substrates[181]. Studies by Goldberg et al.[184] also suggested that human skin taken from an appropriate site may be the best substrate for demonstrating bullous pemphigoid antibodies. They examined the reactivity of three bullous pemphigoid sera on 36 skin specimens obtained from different sites and showed that the highest titres were observed with skin from areas of the body commonly involved with bullous pemphigoid such as flexor arm, flexor thigh and popliteal fossa. An analysis comparing serum titres obtained with flexural skin, monkey oesophagus and guinea pig oesophagus showed normal skin to give titres that were equal or even higher than those noted with monkey or guinea pig oesophagus[184]. The sensitivity of the same substrates for indirect immunofluorescence assay of bullous pemphigoid antibodies was evaluated by Bystryn and Sabolinski[62]. Normal human skin was obtained from different locations on the body. The study included 56 sera containing anti-basement membrane zone antibodies. Similar results between the three substrates were found in 45% of the samples. Variations in reactivity on different substrates was seen in 10 (18%) of cases. More importantly, more than one-third of sera failed to react with one substrate which indicated that the nature of the epithelial substrate has a sig-

nificant effect on the result of indirect immunofluorescence. Although one serum reacted only to human skin, approximately one-third of the samples failed to react against sections of normal skin. This finding is in contrast to the result obtained by Goldberg et al.[184]. However, normal skin used in the study of Bystryn and Sabolinski[62] was randomly collected and not chosen from a flexural area. Guinea pig oesophagus was slightly more sensitive than monkey oesophagus. Fifty-four sera reacted with the former compared to 51 with the latter substrate. However, the sensitivity of the indirect immunofluorescence assay was maximally increased when both substrates were used which raised the proportion of positive sera to 55 out of the 56 samples studied.

Other authors have recommended substrates other than human skin and animal tissue. Gligora[189] studied five cases of bullous pemphigoid and found human tonsil more sensitive than primate oesophagus.

Pemphigus. As in bullous pemphigoid, various investigators have advocated the use of different antigenic substrates for demonstrating intercellular antibodies in pemphigus vulgaris (Table 20). In the original immunofluorescence studies of Beutner and Jordon[41] and Beutner et al.[42], monkey oesophagus and monkey lip were used as substrates to detect pemphigus antibodies. In 1967 Beutner et al.[45] indicated that guinea pig lip and normal skin are suitable substrates for these studies. A study

Table 20

EPITHELIAL SUBSTRATES USED FOR THE DETECTION OF PEMPHIGUS VULGARIS ANTIBODIES

Date	Study Group	Substrates Tested	Recommended Substrate
1969	Chorzelski and Beutner(183)	Monkey, rabbit, guinea pig lip and normal skin	Monkey lip mucosa
1969	Katz et al.(64)	Normal skin, rabbit oesophagus	Normal skin
1978	Weismann et al.(76)	Normal human skin, guinea pig oesophagus	Normal skin
1979	Judd and Mescon(191)	Human foreskin, guinea pig oesophagus, rabbit oesophagus, monkey oesophagus and lip	Monkey oesophagus and lip
1981	Feibelman et al.(182)	Monkey and guinea pig oesophagus	Monkey oesophagus
1982	Acosta and Ivanyi(73)	Human skin and tonsil, monkey oesophagus and lip, guinea pig oesophagus and lip and rabbit lip	Human tonsil and skin
1986	Bystryn and Sabolinski(62)	Normal skin, monkey oesophagus and guinea pig oesophagus	Monkey oesophagus
1987	Kransy et al.(190)	Normal skin; monkey, guinea pig, rabbit and dog oesophagus	Monkey oesophagus
1987	Sabolinski et al.(192)	Monkey and guinea pig oesophagus	Monkey oesophagus
1992	Alkarawi	Normal skin, monkey oesophagus and guinea pig lip	Monkey oesophagus

by Chorzelski and Beutner[183] later demonstrated that considerable disparity may occur with the use of different epithelial substrates in indirect immunofluorescence. They reported two cases of pemphigus vulgaris in which serum testing yielded negative results with human skin, monkey epidermis, rabbit and guinea pig lip tissue whereas high titres were observed when monkey oral mucosa was used. In contrast, others suggested that normal human skin is a more reliable tissue than mammalian epithelia. Katz et al.[64] found that human skin was a more sensitive substrate than rabbit oesophagus and Weissman et al.[76] stated that human skin is a superior substrate compared to guinea pig oesophagus.

The conflicting indirect immunofluorescence results obtained with different epithelial tissue has prompted research to compare the sensitivity of commonly used substrates and determine the most reactive with pemphigus vulgaris antibodies. Feibelman et al.[182] studied the subject of substrate sensitivity by comparing serum titres from 16 patients with active pemphigus using monkey and guinea pig oesophagi. Five of the patients who had shown a positive antibody titre against monkey oesophagus failed to demonstrate activity when guinea pig oesophagus was used. In addition, antibody titres were consistently lower on guinea pig oesophagus. In another study Acosta and Ivanyi[73] confirmed the effect of antigenic substrate on the results of immunofluorescence examination. The

authors compared the sensitivity of different epithelial sources including human skin, human tonsil, monkey oesophagus and lip, guinea pig oesophagus and lip and rabbit lip. Sera from five patients with active pemphigus were used to test the reactivity of substrates. The highest antibody levels were demonstrated when human tissue was used. Although skin and tonsil yielded similar results in terms of antibody titre, greater epidermal and dermal background fluorescence was noted with skin compared to tonsil. Monkey oesophagus and lip produced the best results compared to other animal substrates. The least useful tissues were guinea pig lip and rabbit lip. The high reactivity of pemphigus antibodies with human tonsil has been reported by others[189]. A group of 75 sera with pemphigus antibodies were tested on human skin, monkey and guinea pig oesophagi[62]. Similar results between the three substrates were found in 59% of the samples. Eight sera (11%) showed variable titres on different substrates. However, the most striking finding was the failure of about one-third of the samples to react with any of the substrates. The most reactive tissue was monkey oesophagus with only one serum failing to react to it. Fifty-three (71%) samples were positive on guinea pig oesophagus. The reactivity of human skin was slightly lower than that of guinea pig oesophagus. The low sensitivity of normal skin was probably the result of using specimens with weak antigen expression. The combined use

of monkey oesophagus and guinea pig oesophagus was found to increase the sensitivity of indirect immunofluorescence to 100% in pemphigus. Kransy et al.[190] compared the reactivity of 111 sera from 11 patients with pemphigus on five substrates. Samples were examined on normal human breast skin, monkey oesophagus, guinea pig oesophagus, rabbit oesophagus and dog oesophagus. Many of the sera tested showed variable reactivity on different substrates. The highest frequency of positive sera was obtained using monkey oesophagus. Pemphigus antibodies were detected in 104 (94%) of the sera tested when this substrate was used. Sera from three patients failed to react to guinea pig oesophagus, human skin and dog oesophagus. Samples from three other patients yielded a negative reaction on human skin. The study showed that sera which were consistently negative for pemphigus antibodies on monkey oesophagus were also non reactive on sections from other substrates. In a similar study Judd and Mescon[191] tested 56 pemphigus sera on human foreskin, guinea pig oesophagus, rabbit oesophagus, monkey oesophagus and monkey lip mucosa. There was no significant difference in the frequency of positive results on rabbit, guinea pig oesophagus or human skin. Primate tissues were considered better substrates because of a high rank correlation coefficient. Sabolinski et al.[192] studied 50 sera from patients with various forms of pemphigus on monkey oesophagus and guinea pig oesophagus and confirmed the conclusion of other in-

vestigators that the choice of substrate has a considerable influence on the result of immunofluorescence tests for pemphigus antibodies. Eight of the 50 samples tested failed to react against either monkey oesophagus or guinea pig oesophagus. The authors confirmed previous observations[62] that optimal results are obtained when both substrates are used concomitantly. In addition, Sabolinski et al.[192] investigated whether the reactivity of antibodies with monkey oesophagus or guinea pig oesophagus is related to the clinical form of pemphigus. They showed that pemphigus vulgaris sera can be differentiated from pemphigus foliaceus sera by variations in reactivity on the substrates. Pemphigus vulgaris antibodies produced higher titre and brighter fluorescence on monkey oesophagus whereas sera from pemphigus foliaceus and Brazilian pemphigus foliaceus patients gave more intense reactions on guinea pig oesophagus.

Finally, it is important to emphasize that although the results of the present study are in accordance with previous reports in that antibody titres do vary on different antigenic substrates, false negative results were not observed, i.e. none of the sera tested failed to react against any of the substrates. Thus, the diagnosis would still have been established regardless of the tissue used in the immunofluorescence assay. This investigation confirmed that while monkey oesophagus is the most sensitive substrate for detecting bullous pemphigoid and pemphigus

vulgaris antibodies, guinea pig lip and human skin may be used as reliable alternative tissue for routine immunofluorescence testing if primate oesophagus is not available.

4.2 RESTRICTED DISTRIBUTION OF IgG SUBCLASSES IN BULLOUS PEMPHIGOID AND PEMPHIGUS VULGARIS

Bullous Pemphigoid

In the present study 17 skin biopsy specimens and 25 serum samples from patients with active bullous pemphigoid were tested. Eighty-eight percent of skin specimens contained IgG_4 antibody while IgG_1 was present in 59% of cases. IgG_4 was the only subclass present in five specimens. IgG_3 was the rarest subclass. The third component of complement was observed in 16 (94%) out of 17 biopsies. Serum studies also demonstrated that IgG_4 was the predominant circulating subclass. Only one serum sample was negative for IgG_4 antibody. The ability of bullous pemphigoid antibodies to fix complement in vitro was also determined in this study using indirect complement immunofluorescence. Complement fixing antibodies were detected in 15 (60%) of the 25 cases examined.

Complement fixing IgG subclasses were present in three sera yet they did not fix complement. In addition, some of the complement fixing sera contained only IgG_4.

The demonstration of a predominant IgG_4 response in the skin and serum of bullous pemphigoid patients is in accordance with the results obtained in previous studies.

The distribution of IgG subclasses in various investigations is indicated in Table 21.

Subtyping of anti-basement membrane zone antibodies was first reported in 1973[193]. The authors established that bullous pemphigoid antibodies are heterogenous. They examined serum antibodies from six patients. In three of the six sera tested IgG_4 was the only IgG subclass observed whereas all IgG subclasses were demonstrated in the remaining sera. In a more recent study by Bird et al.[108], the nature of IgG subclass distribution of the antibody in bullous pemphigoid was examined using monoclonal antibodies specific for each of the four subclasses. The study included 24 patients with active disease. The results showed IgG_4 in 23, IgG_1 in 18, IgG_3 in 8 and only three patients were weakly positive for IgG_2. The non-complement fixing subclass, IgG_4, was the only antibody observed in two biopsies which were negative for complement. Circulating IgG antibodies were detected in 20 serum samples. Serum subclass distribution of anti-

Table 21

THE DISTRIBUTION OF IgG SUBCLASSES IN BULLOUS PEMPHIGOID

Date	Author	No. Tested	Specimen	IgG Subclasses G_1	G_2	G_3	G_4
1973	Sams and Schur[193]	6	Serum	3	3	3	6
1986	Bird et al.[108]	24	Skin	18	3	8	23
		20	Serum	16	0	0	20
1986	Flotte and Baird[196]	5	Skin	3	1	0	5
		7	Serum	7	3	3	7
1987	Brooks et al.[194]	9	Serum	3	4	2	9
1989	Kelly et al.[109]	12	Skin	8	1	3	12
		12	Serum	8	0	2	12
1989	Yamada et al.[195]	29	Serum	15	16	8	29
1992	Alkarawi	17	Skin	10	0	3	15
		25	Serum	14	0	4	24

-basement membrane zone antibodies demonstrated the presence of IgG_4 in all 20 sera whereas IgG_1 was detected in 16 samples. Unlike the study of Sams and Schur, IgG_2 and IgG_3 were not observed in any of the sera examined. The predominance of IgG_4 in the serum and tissue of bullous pemphigoid patients was confirmed in another study by Kelly et al.[109] of a group of 12 patients. All skin biopsies showed IgG_4. IgG_1 was present in 8 cases while IgG_2 and IgG_3 were demonstrated in one and three patients respectively. Serum subclass analysis revealed IgG_4 in all samples. IgG_1 was demonstrated in 8 sera whereas IgG_3 was only found in the sera of two patients. IgG_2 was not detected. The IgG subclass distribution reported by Brooks et al.[194] also showed that IgG_4 was the most frequently detected subclass in serum, it being demonstrated in all 9 cases studied. IgG_1 was found in 33%, IgG_2 in 44% and IgG_3 in 22% of cases. Yamada et al.[195] examined the sera of 29 patients with bullous pemphigoid. They confirmed the heterogenous distribution of IgG subclasses in serum. IgG_4 was the predominant anti-basement membrane zone antibody in 29 patients. IgG_1 was detected in 15 cases. Like Brooks et al.[194], the authors found IgG_2 a relatively frequently detected subclass in serum, being observed in 16 cases. Eight samples showed IgG_3 activity. Flotte and Baird[196] evaluated the heterogeneity of IgG response in bullous pemphigoid by direct and indirect immunofluorescence using anti-human mouse monoclonal an-

tibodies against IgG subclasses. The results showed predominance of IgG_4 subclass in circulating and tissue bound antibodies. Hadi et al.[197] studied 50 patients with pemphigoid. IgG_4 was present in most patients with circulating antibodies. IgG_1 was the next most reactive antibody. Sequential serum studies over a period of several years showed two groups of patients. One group consisted of patients with high IgG_4 titre which tended to persist for long periods in spite of clinical improvement and discontinuation of treatment. The second group included patients presenting with low antibody titres that eventually became undetectable within a few months. The authors also found that serum IgG_4 concentration was significantly raised compared to normal controls. Elevated levels of serum IgG_4 were associated with high titres of basement membrane zone antibodies. A correlation between total serum IgG_4 and bullous pemphigoid antibody titres was also noted by others[108].

In contrast to the result obtained in this study, Yamada et al.[195] detected more frequent binding of complement by bullous pemphigoid antibodies in vitro. They observed complement fixing antibodies in 72% of their patients. Furthermore, analysis of the relationship between IgG subclasses distribution and their capability of complement fixation showed that in addition to IgG_4 at least one of the IgG subclasses which are capable of activating complement was also observed in all complement

fixing sera. On the other hand, the non-complement fixing sera were only positive for IgG_4. Thus the results were in accordance with the complement activating characteristics of IgG subclasses. Conversely, the distribution of IgG subclasses in bullous pemphigoid sera examined in this study did not correlate with their complement activating capacity. Although the cause for this discrepancy is not clear, it is possible that complement activation, which requires at least two closely spaced IgG molecules to bind antigen[198], did not occur because only few antigenic sites were available. The demonstration that sera containing only IgG_4 do activate complement has also been reported by Kelly et al.[109]. They detected complement fixing antibodies in 9 out of 12 patients including serum from one patient in whom only IgG_4 was present. Unlike other investigators, Brooks et al.[194] detected complement fixing antibodies in only 3 (33%) of 9 pemphigoid sera. The authors did not indicate whether the result correlated with the IgG subclass findings.

The predominance of IgG_4 antibody in the sera and skin of pemphigoid patients is a surprising finding because it does not correlate with its distribution in normal serum which is 3-4% of total IgG[114]. In contrast, IgG_1, which represents 64-70%[114], was consistently present in lower quantities.

Although immunoglobulin responses may be seen in all four subclasses, a restricted subclass response is more commonly observed in certain disorders including autoimmune conditions. It has been shown that IgG_4 was the predominant subclass in the autoimmune blistering disorders pemphigus[107] and epidermolysis bullosa acquisita[199]. High IgG_4 anti-thyroglobulin titres in patients with autoimmune thyroiditis have been reported[200]. Antibodies to factor VIII are primarily IgG_4[201]. This subclass has also been recognized as the major IgG antibody against a variety of antigens such as grass pollen allergens[106] and certain food antigens[202].

The cause of the prominent production of IgG_4 in bullous pemphigoid has not been established. It is possible that it represents an acquired phenomenon related to the nature of the antigen. Conversely, this restricted subclass response may be the result of specific genetic factors[114]. It has been speculated that continued antigenic stimulation affects the normal distribution of IgG subclasses and leads to an IgG_4 restricted response[203]. However, sequential studies of IgG_1 and IgG_4 in bullous pemphigoid serum did not reveal any major change in the pattern of IgG subclasses[197].

In addition to being present in low concentration in normal serum, IgG_4 is unique compared with other IgG subclasses because it does not activate complement by the classical pathway[90]. It is also doubtful whether IgG_4 has

the capacity to activate complement via the alternative pathway[204]. Thus, the prominent IgG_4 response was also an unexpected finding since almost all patients tested in this study demonstrated C_3 deposits in the basement membrane zone. Furthermore, five of the 16 cases with complement deposits had an IgG_4 restricted response. Similar findings were observed by Kelly et al.[109]. They reported that IgG_4 was the only subclass detected in the perilesional skin of two bullous pemphigoid patients despite the presence of tissue fixed C_3 in all 12 cases studied. Conversely, others[108,195] have shown that pemphigoid cases with complement deposits were always associated with one of the IgG subclasses which are characterized by their ability to activate complement.

Involvement of the complement system in the pathogenesis of bullous pemphigoid has been suggested. However, the precise role that complement plays in the development of the disease has not been fully determined.

It has been shown that circulating anti-basement membrane zone antibodies in some patients lack the capacity to activate complement. Sams and Schur[193] demonstrated that non-complement fixing sera were composed only of IgG_4 whereas sera containing complement fixing antibodies were found in the subclasses G_1, G_3 and G_4.

Roberts et al.[205] showed a correlation between the distribution of IgG subclasses in in vitro complement studies and the histologic picture in two distinct types

of glomerular pathology in systemic lupus erythematosus. IgG_1 and IgG_3, which have the greatest capacity to fix complement, were the dominant IgG subclasses in proliferative glomerulonephritis. In vitro complement studies were positive in all cases. Conversely, the poor complement fixing subclasses, IgG_2 and IgG_4, were detected in membranous glomerulonephritis. Antibodies in this form generally failed to fix complement in vitro. The authors suggested that the difference in the biological property of complement fixation of these antibodies provides an explanation for the inflammatory histologic picture of the proliferative form and the contrasting bland morphology of membranous glomerulonephritis.

In support of a pathogenic role for complement in bullous pemphigoid is the detection of C_3 in a linear pattern at the basement membrane zone in all patients with active disease[6]. Immunoelectron microscopy studies show C_3 deposits localized in the lamina lucida which correspond to the ultrastructural site of the blister in pemphigoid[206]. This is in addition to the finding of other components of the classical and alternate pathways at the dermal epidermal junction[207]. Dahl[208] demonstrated deposition of membrane attack complex (C_5-C_9) in perilesional skin which established that complement activation proceeds to the terminal stage. Furthermore, in vitro studies[209] have shown that bullous pemphigoid antibodies are capable of activating complement via the

classical and alternate pathways. Local activation of complement is demonstrated by the depressed levels of total haemolytic complement and individual components in blister fluid complement[210]. Gammon et al.[211] described an in vitro model for bullous pemphigoid which demonstrated a functional interaction between bullous pemphigoid antibodies, complement and peripheral blood leukocytes. The leukocyte attachment method involved the incubation of cryostat sections of normal human skin with complement fixing bullous pemphigoid antibodies, viable leukocytes and fresh normal human serum as a complement source. Subsequently, leukocytes migrated and attached to the basement membrane zone. In addition, prolonged incubation of the activated leukocytes was associated with focal areas of basement membrane zone separation as seen in early lesions of bullous pemphigoid. The importance of serum complement in the process of leukocyte attachment and dermal epidermal separation was shown by the significant reduction of leukocyte attachment and dermal epidermal separation when the complement source was omitted. In a study by Naito et al.[212], human skin explants were cultured with sera, IgG fractions and blister fluid from patients with bullous pemphigoid. Dermal epidermal separation occurred with blister fluid. The significance of complement in this experiment was demonstrated by the lack of dermal epidermal separation when complement inactivated by heat or anti-serum to

complement was used. In another report Naito et al.[213] demonstrated dermal epidermal separation in vivo by injecting concentrated bullous pemphigoid serum or IgG fraction from pemphigoid patients into the skin of guinea pigs. The development of blisters was completely inhibited when bullous pemphigoid IgG was injected into C_3 inactivated guinea pigs. However, dermal epidermal separation was noted in some C_4-deficient animals. The results suggested that the alternative pathway may participate in the formation of bullous pemphigoid blisters.

Sams and Gammon[214] proposed that the process of blister formation in bullous pemphigoid is initiated by the binding of complement-activating IgG antibodies to the bullous pemphigoid antigen with subsequent activation of complement and the production of many inflammatory mediators including the generation of the anaphylotoxins C_{3a} and C_{5a}. As a result, mast cells degranulate and release histamine and chemotactic factors such as eosinophil chemotactic factor of anaphylaxis and neutrophil chemotactic factor which lead to the recruitment and activation of eosinophils and neutrophils. The inflammatory cells adhere to the basement membrane zone and liberate proteolytic enzymes that cause dermal/epidermal separation.

The role of mast cells in bullous pemphigoid has been evaluated by sequential histologic examination. Wintraub et al.[215] presented a morphologic evidence for the release

of mast cell products and showed that the cells are involved in the initial stages of blister formation in bullous pemphigoid. This was based on histological examination of lesions obtained at different clinical stages. Early erythematous lesions showed hypogranulated mast cells in the papillary dermis whereas more advanced lesions were associated with more extensive mast cell hypogranulation. The events that cause mast cells to degranulate are unclear. IgE antibodies after binding with antigen can trigger mast cell degranulation. Although deposits of IgE have been detected in the basement membrane zone of patients with pemphigoid, this occurs only occasionally, which suggests that IgE is unlikely to be a major mediator of mast cell activation. It is possible that complement anaphylotoxins cause the degranulation of mast cells. However, in view of some doubts over the role of complement[216], the existence of non-complement fixing sera[193] and the frequent detection of IgG_4 subclass in this study and previous investigations, it is conceivable that the inflammatory response in bullous pemphigoid may occur through other mechanisms.

IgG_4 is an antibody which does not have the capacity to activate complement but has been shown to have homocytotropic properties for mast cells[102]. Thus, the interaction of IgG_4 antibodies with mast cells in the skin[108] may represent an alternative or additional

mechanism of tissue injury leading to mast cell degranulation, inflammation and blister formation in bullous pemphigoid.

Pemphigus Vulgaris

In this study 9 skin biopsy specimens and 17 serum samples from patients with active pemphigus were examined. All skin and sera had IgG. The predominant IgG subclass in both skin and serum was IgG_4, which was detected in 100% of cases. Seven (78%) of the biopsies studied had IgG_4 as the only subclass present. C_3 component of complement was detected in all skin specimens. IgG_4 was the only subclass observed in 10 (59%) of the 17 sera tested. The complement fixing capability of intercellular antibodies in the 17 sera was determined by indirect complement immunofluorescence. The results of the in vitro complement test appeared to correlate well with the distribution of IgG subclasses. All seven sera containing complement fixing antibodies were also positive for IgG_1 which is characterized by a strong capacity to activate complement.

The frequency of IgG subclasses reported by different investigators is shown in Table 22. The IgG subclass distribution in pemphigus serum was first determined by Sams and Schur[193] using stepwise immunoglobulin elution from diethylaminoethyl cellulose columns and immunofluorescence methods. The IgG subclasses were localized using

Table 22

THE DISTRIBUTION OF IgG SUBCLASSES IN PEMPHIGUS VULGARIS

Date	Author	No. Tested	Specimen	IgG Subclasses G_1	G_2	G_3	G_4
1973	Sams and Schur[193]	3	Serum	3	3	3	3
1987	Brooks et al.[194]	15	Serum	11	9	7	5
1988	Jones et al.[107]	10	Serum	9	0	1	10
1989	David et al.[217]	13	Skin (active disease)	13	7	10	11
		14	Skin (remission)	7	2	4	11
1989	Yamada et al.[195]	27	Serum	7	6	5	27
1989	Kelly et al.[109]	10	Skin	9	0	2	10
		10	Serum	9	1	1	10
1992	Alkarawi	9	Skin	3	0	0	9
		17	Serum	8	0	0	17

monospecific antisera to IgG and the different subclasses. The results showed that serum IgG from three patients was composed of all four subclasses. This is in contrast to the results obtained in this study where IgG_2 and IgG_3 were absent from all skin and sera tested. The distribution of IgG subclasses was determined by Jones et al.[107] in a study of 10 pemphigus sera using monoclonal antibodies against human IgG subclasses. They found that the IgG antibody response was composed mainly of the IgG_1 and IgG_4 subclasses. IgG_4-specific autoantibodies were demonstrated in all 10 sera with antibody levels up to 1:320. IgG_1 antibodies were found in 9 serum samples but at lower titres. IgG_3 was detected in the serum of one patient only after isolation of IgG using ion exchange chromatography. IgG_2 was not detected in any sample. The predominance of IgG_4 in the sera of pemphigus patients was confirmed by Yamada et al.[195]. In a study of 27 patients with various types of active pemphigus the intercellular antibody activity was detected in all four IgG subclasses. IgG_4 was detected in the sera of all patients. IgG_1 was present in 7 sera whereas IgG_2 and IgG_3 were observed in 6 and 5 cases respectively. In another report Kelly et al.[109] analysed the pattern of IgG subclasses in the serum and skin of 10 pemphigus vulgaris patients. IgG_4 was observed in all 10 biopsies examined. IgG_1 and IgG_3 were positive in 9 and 3 cases respectively whereas IgG_2 was not detected in any of the cases studied. IgG_4 and IgG_1

subclasses were also the predominant intercellular antibodies in serum. IgG_2 and IgG_3 were rarely detected. A heterogenous distribution of IgG subclasses in the sera of patients with pemphigus vulgaris was reported by Brooks et al.[194]. In contrast to other studies, IgG_1 was the most commonly detected subclass whereas IgG_4 was the least encountered antibody. Examination of 15 patients showed that IgG_1 was found in 11, IgG_2 in 9, IgG_3 in 7 and IgG_4 in only 5 patients. David et al.[217] compared the distribution of IgG subclasses in the perilesional skin of two groups of pemphigus vulgaris patients. The first included 13 patients with active disease and the other group consisted of 14 patients in remission. IgG_1 was detected in all patients with active pemphigus while IgG_4 was present in 11 of the 13 biopsy specimens studied. IgG_2 and IgG_3 were seen in 7 and 10 cases respectively. IgG_4 was the predominant subclass in patients who were in clinical remission, being observed in 11 of 14 patients. Complement was found in 11 patients in the first group but detected less frequently among patients in remission. Analysis of the results showed that the frequency of IgG_1, IgG_3 and C_3 among patients in remission decreased significantly compared with active disease. It was concluded that C_3 and IgG_3 are possible indicators of a state of remission whereas IgG_1 may be considered a marker of disease activity.

Studies of IgG subclasses in endemic pemphigus foliaceus showed results which were generally similar to those in other types of pemphigus. IgG_4 is the predominant antibody in the skin and serum of patients with this disorder. The next most reactive antibody is IgG_1. The remaining IgG subclasses are detected only occasionally in both circulating and tissue bound antibodies[123].

Several investigators have studied the complement binding capability of IgG subclasses in pemphigus. Yamada et al.[195] found complement fixing antibodies in only 6 of 27 pemphigus sera. Three samples contained one of the complement fixing IgG subclasses (G_1-G_3) as well as IgG_4. Conversely, the result of the remaining three sera was not in accordance with the complement fixing characteristics of IgG subclasses because the three samples were only positive for IgG_4. Furthermore, in the 21 sera which did not contain complement fixing antibodies only IgG_4 was present in 13 samples, whereas in the remaining 8 sera subclasses with complement fixing ability were detected in addition to IgG_4. This finding is similar to that reported in the study of Sams and Schur[193] where three sera from patients with active pemphigus failed to fix complement in spite of the presence of the complement fixing subclasses G_1, G_2 and G_3. Brooks et al.[194] studied the complement binding activity in their patients and found that two-thirds (10 out of 15) of the pemphigus sera

tested bound complement in vitro. This appeared to correlate with the frequency of IgG_1 (11 out of 15) which is an efficient complement fixer. The authors, however, did not point out the distribution of IgG subclasses in the sera with complement fixing antibodies.

It has been proposed that pemphigus antibodies have an important role in the pathogenesis of the disease. The suggestion that intercellular antibodies are involved in the development of pemphigus lesions has been based on different clinical and laboratory observations. These antibodies are deposited in the skin of all patients[67]. They are also present in the sera of most patients and the titre tends to correlate with disease activity[70]. Removal of pemphigus antibodies induces a state of remission[218]. Neonates of mothers with pemphigus may have the disease too. It gradually improves with the disappearance of the placentally transferred maternal pemphigus antibodies[219]. In addition, there are in vitro studies which suggest that circulating pemphigus vulgaris antibodies may be pathogenic. Michel and Ko[220] showed that serum antibodies can cause acantholysis. When serum is incubated in vitro with normal skin organ cultures, antibody binds to the epidermal intercellular areas in the tissue explant. This was associated with suprabasal acantholysis. Schiltz and Michel[221] used explants of normal skin in organ culture and identified IgG fraction from pemphigus serum as the element responsible for acantholysis. Further studies[222]

demonstrated that pemphigus antibodies cause epidermal cell detachment when added to monolayers of epidermal cells in culture. Farb et al.[222] also showed that pemphigus antibodies elicited the release of proteases from epidermal cells in culture. In a more recent study Hashimoto et al.[223] suggested that plasminogen activator is the protease secreted by epidermal cells treated with pemphigus IgG. Furthermore, the authors found that acantholysis does not occur if protease inhibitors are added to the cell cultures. It was therefore concluded that acantholysis in pemphigus may be mediated by the plasminogen plasmin system. The involvement of intercellular antibodies in the pathogenesis of pemphigus is also supported by in vivo studies. The disease has been induced in neonatal mice by the injection of IgG fractions of serum from patients with pemphigus[224]. Using the neonatal mouse model, Anhalt et al.[225] found that the addition of dexamethasone can significantly reduce the release of pemphigus IgG induced plasminogen activator by epidermal cells. Treatment with dexamethasone, however, did not inhibit the development of blisters.

It is not clear whether activation of complement system is necessary for the induction of pemphigus. In addition to C_3, other complement components have been identified in pemphigus skin including C_{1q} and C_4[226,227]. Properdin has also been observed in some cases, which suggests a degree of complement activation by the alternative

pathway[226]. The involvement of the complement system is supported by the presence of low total haemolytic complement in pemphigus blister fluid compared to serum levels[228]. Activation of complement is also suggested by the reduced levels of the early classical pathway components C_1, C_q and C_4[229].

Although intercellular antibody activity has been observed in IgG subclasses that are usually associated with complement fixation, the initial attempts of Jordon et al.[230] to demonstrate that pemphigus antibodies are capable of fixing complement were unsuccessful. However, subsequent in vitro complement studies by Nishikawa et al.[231] demonstrated that some pemphigus antibodies will fix complement to human skin. Although there are indications that complement may have a role in the pathogenesis of pemphigus, in vitro studies have demonstrated that acantholysis can be induced by pemphigus sera devoid of complement[221]. A study by Anhalt et al.[224] provided further evidence that complement activation is not a prerequisite for blister formation. The authors showed that $F(ab)_2$ fragments of human pemphigus antibodies do produce pemphigus lesions in the neonatal mouse model despite the lack of the complement fixing Fc portion. In addition, when pemphigus vulgaris antibodies were injected into normal and genetically deficient C_5 neonatal mice extensive blistering was observed in all animals. Acantholysis was also noted in controls and mice pretreated with cobra

venom factor to deplete C_3 when large doses of pemphigus vulgaris IgG were used. However, a significant decrease in disease activity was noted when lower doses of antibody were administered. The result suggested that, under certain conditions, complement activation may have an amplifying role in the development of pemphigus lesions.

The predominance of IgG_4 subclass in the serum and skin in pemphigus vulgaris led to the suggestion that it may contribute to the pathogenesis of this disorder[232]. This IgG subclass has been implicated as an aetiological factor in the pathogenesis of allergic disorders. High concentrations of IgG_4 have been observed in atopic dermatitis[120,233] and asthma[121]. In addition, various allergens may be associated with elevated levels of IgG_4[106,202]. It has been suggested that IgG_4 can sensitize leukocytes for the release of histamine and thus play a role in hypersensitivity reactions. On the other hand, a blocking effect of IgG_4 has been proposed[105,106,234]. This occurs by the binding of IgG_4 and allergen complexes on effector cells, thus interfering with the mechanisms which lead to histamine release. A protective role for IgG_4 has also been suggested in the case of immunotherapy. Aalberse et al.[203] found that the initial response to the honey bee venom phospholipase A is predominantly of the IgG_1 subclass whereas repeated ex-

posure to the antigen was associated with a change in the antibody distribution with antibody activity being predominantly of the IgG_4 subclass.

In their study of pemphigus vulgaris patients, David et al. speculated that IgG_4, which was the most common subclass in patients in remission, may act as a blocking antibody to other IgG subclasses. However, a blocking or protective function of IgG_4 in pemphigus has not been proved. In fact, there is in vivo evidence supporting a pathogenic role for IgG_4 antibodies in one type of pemphigus, endemic pemphigus foliaceus. The pathogenicity of this subclass was established when passive transfer of IgG_4 from patients to a neonatal mouse model was associated with a reproduction of the clinical histological and immunological features of the human disease[123]. It therefore seems likely that IgG_4 antibodies may also contribute to the development of tissue injury in pemphigus vulgaris.

4.3 IMMUNOFLUORESCENCE AND WESTERN IMMUNOBLOTTING STUDIES OF ANTI-BASEMENT MEMBRANE ZONE ANTIBODIES

The differentiation between bullous pemphigoid and other bullous diseases may be more difficult than previously believed, particularly in patients with epidermolysis bullosa acquisita. Although the specificity of circulating anti-basement membrane zone antibodies in each of the two disorders has been shown to be distinct, an overlap between the clinical histologic and routine immunofluorescence features are similar and can cause problems.

Studies of patients with epidermolysis bullosa acquisita diagnosed on the basis of specific criteria[28,124] showed that this disorder may be confused with other blistering diseases, especially bullous pemphigoid. Gammon et al.[29] reported a patient with generalized bullous eruption indistinguishable from bullous pemphigoid but with immunoelectron microscopy features of acquired epidermolysis bullosa. Classical features of epidermolysis bullosa acquisita eventually developed. In another report Gammon et al.[235] described cases in which clinical manifestations of bullous pemphigoid or, less commonly, cicatricial pemphigoid were observed on presentation or during the course of the illness whereas immunoelectron microscopy and immunoblotting results were consistent with a diagnosis of epidermolysis bullosa acquisita. These observations

provide evidence that the dependence on clinical and immunopathologic features to distinguish between bullous pemphigoid and epidermolysis bullosa acquisita may lead to incorrect diagnoses. The establishment of a definite diagnosis is of particular importance in view of the differences in the natural history and response to therapy between the two disorders. Epidermolysis bullosa acquisita typically runs a chronic and protracted course with only limited response to treatment. In contrast to bullous pemphigoid, which is relatively easy to control by corticosteroids, epidermolysis bullosa acquisita usually requires higher initial doses and longer maintenance of corticosteroids in addition to immunosuppressive or cytotoxic agents[143].

In the present study basement membrane zone antigens were localized using sodium chloride-separated skin as a substrate. The investigation included one epidermolysis bullosa acquisita and 27 bullous pemphigoid sera. All pemphigoid sera produced an epidermal fluorescence pattern whereas the epidermolysis bullosa acquisita serum stained the dermal aspect of the split. In addition, testing for type IV and type VII collagen showed that they were localized on the floor of separated skin. It was noted that the incubation time required to separate the skin was approximately 96 hours at 4°C whereas a duration of only 48 hours at room temperature was necessary before separation

was achieved. This may have been due to continued protease activity allowed for by the higher temperature[236].

The same technique was also used to separate perilesional skin biopsies obtained from bullous pemphigoid patients. Seven specimens were stained by FITC conjugated IgG and C_3. Immunoreactants were detected on the epidermal side of the split in six specimens whereas a split biopsy specimen from one patient showed IgG and C_3 staining on both the epidermal and dermal sides of the separation. Western immunoblotting was also performed utilizing circulating anti-basement membrane zone antibodies from the sera of patients with bullous pemphigoid and epidermolysis bullosa acquisita. Five of 7 pemphigoid sera which stained the epidermal aspect of separated skin reacted by immunoblotting. An epidermolysis bullosa acquisita serum did not react with any band. Four bullous pemphigoid sera recognized a 220 KD protein whereas one serum showed reactivity with a 180 KD antigen.

Adult human skin may be separated at the dermal epidermal junction by different methods including cold sodium chloride, cold trypsinization, induction of a suction blister and phosphate buffered saline. Various basement membrane components were localized by indirect immunofluorescence on skin separated by these techniques. Laminin and type IV collagen, which are localized in the lamina lucida and lamina densa respectively, are usually

detected along the dermal edge and around dermal blood vessels. Bullous pemphigoid antibodies reacted against split skin consistently produced staining of the roof of the split regardless of the separation method used[237]. In addition, it has been suggested that the ultrastructural binding sites of circulating anti-basement membrane zone antibodies of other bullous disorders can be determined by indirect immunofluorescence using sodium chloride separated skin as a substrate[238].

Treatment of normal skin with 1M sodium chloride causes separation of the basement membrane through the lamina lucida[237]. Thus, all the antigenic compounds within or beneath the lamina densa are localized on the dermal side of the separated skin. Consequently, binding of bullous pemphigoid anti-lamina lucida antibodies (as determined by immunoelectron microscopy) would be expected to occur on the epidermal side of the split whereas epidermolysis bullosa acquisita sera, which contain anti-sublamina densa antibodies, would react with the dermal aspect of the separation. Based on these data, Gammon et al.[238] confirmed and extended previous observations by using skin, separated by sodium chloride at 4°C, to localize the bullous pemphigoid and epidermolysis bullosa acquisita antigens. Antibodies from a reference bullous pemphigoid serum reacted with the epidermal aspect of the split whereas an epidermolysis bullosa acquisita reference serum produced a staining pattern restricted to the dermal

side of the split. The authors also used this method to detect anti-basement membrane zone antibodies in the sera of patients suspected of having bullous pemphigoid on the basis of clinical, histological and routine immunofluorescence features. Six of 51 sera showed binding on the dermal side of the split and immunoelectron microscopy studies revealed that these samples contained anti-sublamina densa antibodies which are different from the anti-lamina lucida antibodies of bullous pemphigoid. One case of epidermolysis bullosa acquisita showed an epidermal staining pattern and proved to have lamina lucida deposits. A combined epidermal dermal pattern, exhibited by 9 sera, was associated with lamina lucida antibodies.

Gammon et al. therefore proposed that a significant proportion of patients thought to have bullous pemphigoid have epidermolysis bullosa acquisita. Furthermore, they indicated that a bullous pemphigoid-like presentation of acquired epidermolysis bullosa may be seen in up to 50% of patients[235,238]. The same authors determined the ultrastructural binding site of 34 additional patients thought to have bullous pemphigoid using indirect immunofluorescence and split-skin. The results showed that 5 sera produced a dermal fluorescence pattern consistent with a diagnosis of epidermolysis bullosa acquisita which was subsequently confirmed by indirect immunoelectron

microscopy. Thus they suggested that about 10% of patients originally diagnosed as bullous pemphigoid may actually have epidermolysis bullosa acquisita[238].

Logan et al.[239] evaluated the use of indirect immunofluorescence with the split-skin technique as an alternative method to immunoelectron microscopy for determining the site of immune deposits. In contrast to the findings of Gammon et al., they suggested that patients with dermal staining pattern on sodium chloride separated skin do not invariably have epidermolysis bullosa acquisita. The authors examined a group of 228 sera from cases previously diagnosed as bullous pemphigoid. Nine samples showed a linear pattern of fluorescence along the dermal edge of the separation. The remaining sera produced an epidermal or a combined pattern. When the nine sera were tested by indirect immunoelectron microscopy only three sera showed immune deposits within or beneath the lamina densa. The immune reactants in the other six samples were observed within or above the lamina lucida. Thus there was no compatibility between the immunofluorescence reaction pattern and immunoelectron microscopy findings in these cases. In addition, clinical details available from six of the nine cases were not consistent with a diagnosis of epidermolysis bullosa acquisita. The authors concluded that the split skin tech-

nique was unhelpful in identifying cases of epidermolysis bullosa acquisita incorrectly diagnosed as bullous pemphigoid.

In another study Kelly et al.[236] examined 20 bullous pemphigoid sera on sodium chloride-separated skin. The majority of samples produced an epidermal or combined pattern. The dermal pattern was observed in three cases. Although immune deposits were not localized by immunoelectron microscopy, the authors felt that the clinical features of the three cases were not suggestive of epidermolysis bullosa acquisita.

Conversely, others[240,241] have confirmed that indirect immunofluorescence with split-skin is a useful method to distinguish between anti-bullous pemphigoid and anti-epidermolysis bullosa acquisita antibodies. Zhu et al.[240] presented data supporting the findings of Gammon et al[238] that patients showing a dermal staining pattern may have epidermolysis bullosa acquisita. However, they suggested that the proportion of patients with basement membrane antibodies who may have acquired epidermolysis bullosa rather than bullous pemphigoid is smaller than that previously reported. The authors examined 100 serum samples with anti-basement membrane zone antibodies on split skin. Three sera which were obtained from patients clinically diagnosed as bullous pemphigoid or cicatricial pemphigoid demonstrated fluorescence on the dermal side of the split. Two of these sera reacted with the epider-

molysis bullosa acquisita antigen on Western immunoblotting assay. In another report, Fine et al.[241] observed a dermal pattern when sera from patients diagnosed as epidermolysis bullosa acquisita were tested on split skin. Direct immunoelectron microscopy of perilesional skin showed immunoreactants to be associated with the lamina densa and sublamina densa areas, thus confirming the diagnosis of epidermolysis bullosa acquisita.

McCuaig et al.[242] suggested that the split skin technique may also be a useful diagnostic procedure in children with bullous disorders. They reported a rare childhood case of epidermolysis bullosa acquisita that was originally diagnosed as hereditary epidermolysis bullosa. The correct diagnosis was suggested by indirect immunofluorescence using split-skin and was subsequently confirmed by specialized techniques. It is therefore recommended that this procedure be part of the immunologic evaluation of children with mechanobullous skin disease.

In addition to the value of sodium chloride separated skin in the differentiation between anti-lamina lucida and anti-sublamina densa antibodies, the substrate appears to increase the sensitivity of indirect immunofluorescence for the detection of anti-basement membrane zone antibodies[236,238,241]. The improvement in the sensitivity of split skin may be due to exposure of antigenic sites during the separation process. Low background fluorescence, possibly due to the removal of dermal gam-

maglobulins, may have contributed to the increased ability to detect these antibodies[238]. A similar observation was noted in the present study. Higher antibody titres were obtained with separated skin compared to normal intact skin.

The results of various investigations have demonstrated that the split-skin technique is a useful diagnostic procedure in bullous disorders. In addition, the method is relatively simple and can be performed at any immunofluorescence laboratory which is in contrast to the technically difficult and time-consuming immunoelectron microscopy and immunochemical methods. However, the split skin technique is only applicable in cases associated with circulating anti-basement membrane zone antibodies. This has led some authors[239] to suggest immunofluorescence mapping as an alternative method in patients lacking serum antibodies. The method involves localization of the ultrastructural level of skin cleavage by mapping basement membrane antigenic determinants. Electron microscopy studies have shown that blister formation in epidermolysis bullosa acquisita occurs beneath the lamina densa[124,143]: thus this structure remains in the roof of the blister. In contrast, skin cleavage in bullous pemphigoid develops within the lamina lucida[55], which leaves the lamina densa in the floor of the blister. These levels may be determined by the use of antibodies against normal antigenic components of the basement

membrane such as laminin and type IV collagen. The blister level can be established by evaluating whether the roof or the floor is stained by the antibodies. In bullous pemphigoid the labelling pattern for both laminin and type IV collagen is on the floor of the blister whereas in epidermolysis bullosa acquisita the staining is noted on the roof of the blister[243]. However, others suggested that this method may give inaccurate results in some case of epidermolysis bullosa acquisita. Fine et al.[241] studied 10 patients with this disorder in whom the diagnosis was confirmed by direct immunoelectron microscopy. However, most skin specimens examined showed an intralamina lucida, rather than sublamina densa blister. Consequently, immunomapping showed the immune deposits on the floor of the blister. The authors concluded that routine electron microscopy and immunomapping studies of patients' blistered skin are not reliable diagnostic methods in epidermolysis bullosa acquisita.

In cases where circulating antibodies are absent, direct immunofluorescence on sodium chloride separated skin biopsies has been shown to be a useful technique in delineating cases of bullous pemphigoid from epidermolysis bullosa acquisita. The method involves separating patients' perilesional skin using 1M sodium chloride. The separated skin is then used as a substrate for standard direct immunofluorescence to identify the level of antibody deposition. Gammon et al.[244] showed that in bul-

lous pemphigoid the immune reactants are found on the epidermal side or both sides of the split whereas in epidermolysis bullosa acquisita the immune deposits are associated with the dermal aspect of the separation. Wuepper[245] tested stored frozen biopsies by the direct immunofluorescence separation method and confirmed the usefulness of the procedure in differentiating between anti-lamina lucida and anti-sublamina densa antibodies. These results indicate that the technique is reliable for diagnosing epidermolysis bullosa acquisita and distinguishing it from bullous pemphigoid without requiring the presence of circulating antibodies. It must be stressed, however, that when this method is used accurate results can be obtained only if it is established that separation has taken place through the lamina lucida. Gammon et al.[244] emphasized that this must be confirmed by immunofluorescence mapping because of the possibility of an atypical split occurring with tissue from patients with epidermolysis bullosa acquisita which could lead to inaccurate localization of the immune deposits.

In addition to the split skin techniques described above, a reliable differentiation between bullous pemphigoid and epidermolysis bullosa acquisita can be achieved by Western immunoblotting. In recent years many investigations have been performed to explore the characteristics of the normal antigenic components reactive with anti-lamina lucida and anti-sublamina densa antibodies.

In bullous pemphigoid circulating anti-basement membrane zone antibodies recognize a specific non-collagenous glycoprotein in epidermal extracts which are separated by SDS-PAGE and then electrophoretically transferred to nitrocellulose paper[142]. The antigen is not reactive with normal sera or with antibodies from patients with other bullous disorders, which indicates that Western immunoblotting is a useful method for confirming the diagnosis of bullous pemphigoid.

The use of Western immunoblotting to investigate the bullous pemphigoid antigen was originally described by Stanley et al.[142]. Their results showed that a single 220-240 KD bullous pemphigoid antigen was regularly detected in all immunoblotting studies. However, other authors in subsequent studies presented evidence of bullous pemphigoid antigen heterogeneity at the molecular level. Labib et al.[246] used Western immunoblotting, with epidermal extracts prepared from heat separated epidermis, to determine the proteins reactive with anti-basement membrane zone antibodies from 28 patients with bullous pemphigoid. Proteins with different molecular weights (240 KD, 200 KD, 180 KD, 97 KD and 77 KD) were observed but two particular bands were most often detected by the sera tested. The 240 KD antigen and the 180 KD antigen reacted with 43% and 29% of the samples respectively. The authors suggested that the smaller molecular weight proteins detected in their study do not represent

proteolytic degradation products of larger antigens because, although they were uncommonly observed, they produced intense staining reactions. In addition, they attributed their ability to detect the 180 KD antigen, which was not previously reported to the use in all experiments of a panel of protease inhibitors during the preparation of epidermal extracts.

Heterogeneity of the bullous pemphigoid antigen was first suggested by Zhu and Bystryn[181] on the basis of variations in antigen expression, as determined by indirect immunofluorescence between skin specimens obtained from different individuals. Analysis of the reactivity patterns indicated that there are two groups of bullous pemphigoid antigen. The first is a major antigen reactive with antibodies present in the majority of patients with bullous pemphigoid. The other group consists of antigens which are expressed in some individuals. Antibodies reactive with these 'minor' antigens are only present in a proportion of bullous pemphigoid patients. The same group[247] used Western immunoblotting to confirm that bullous pemphigoid antibodies are heterogenous. In a study of 38 patients they demonstrated that approximately 90% of patients had antibodies to a major bullous pemphigoid antigen with a molecular weight of 230 KD. Antigens with molecular weights of 200 KD and 180 Kd were detected in up to 75% of patients whereas 11% of sera contained antibodies that reacted solely to a 160 KD protein. This

molecular heterogeneity was also observed by other investigators. Bernard et al.[248] reported the detection of various antigens by 30 bullous pemphigoid sera. The most commonly detected antigens were the 220 KD protein and a 165 KD protein. Bands with a molecular weight of 190 KD and 97 KD were also detected by some antibodies. Interestingly, the study included sera which were negative by indirect immunofluorescence on standard substrates and sodium chloride separated skin but produced a positive result by immunoblotting. Fourteen of 29 of these sera contained antibodies that recognized the 220 KD and/or the 165 KD antigen. In contrast, some sera which contain bullous pemphigoid antibodies, demonstrable by indirect immunofluorescence, may not be detected by the immunoblotting procedure. Such sera represented 10% of the total samples assayed by Zhu et al.[240]. It has previously been suggested that there is rough correlation between the indirect immunofluorescence titre of bullous pemphigoid sera and their reactivity by the immunoblotting assay[246]. However, low serum antibody titre was excluded by Zhu as a cause of negative immunoblotting because, as noted in the present study, antibody levels in the sera that failed to stain any bands were comparable to those in other samples which recognized specific bullous pemphigoid antigens. Zhu et al. also ruled out the possibility of degradation or loss of antigen during the extraction process since other simultaneously tested sera resulted in positive im-

munoblots. Conversely, other studies[249] have suggested that the denaturing of bullous pemphigoid antigen (necessary for SDS-PAGE) may lead to complete or partial loss of reactivity with some sera. Mueller et al.[249] could not demonstrate antigen heterogeneity but confirmed that the 230 KD antigen is a frequently detected protein by Western immunoblotting. The authors investigated whether the antibodies reacting with the 230 KD antigen are identical to the bullous pemphigoid antibodies that are detected by indirect immunofluorescence. They affinity purified the IgG bullous pemphigoid antibodies on the 230 KD band and then used these antibodies for immunofluorescence which showed linear binding along the basement membrane zone.

Immunoprecipitation methods were shown by Mueller et al. to be more sensitive than immunoblotting to identify the 230 KD antigen. In addition, they noted that some sera precipitated a 166 KD antigen. The authors suggested that it may be a degradation product of the 230 KD antigen and that it may be identical to the 180 KD antigen described by Labib et al.[246]. However, none of the sera tested recognized either a 166 KD or a 180 KD band by immunoblotting. Mueller et al. used PMSF and EDTA as protease inhibitors during the epidermal extract preparation which may not have been sufficient to prevent proteolysis. In contrast, Labib et al. used additional

inhibitors to different groups of proteases which may account for their ability to detect the 180 KD and the smaller molecular weight proteins.

It has been proposed that the 160-165 KD band detected in some studies corresponds to the 180 KD antigen and that the disparity in molecular weight determination may have been the result of differences in the extraction procedure or the standard markers used[248]. However, Zhu et al.[247,250] regarded the 160 KD band to be distinct from the 180 KD and 230 KD antigens because all have been detected on the same immunoblot and sera reactive mainly with the 160 KD antigen did not recognize the 180 KD protein sera while antibodies directed against the 180 KD antigen did not react to the 160 KD antigen. In addition, they suggested that the 180 KD protein may be a breakdown product of a larger molecule since it was not regularly detected in different extracts. To study the effect of proteolysis on the different antigens, Zhu et al.[247,250] prepared blots using epidermal extracts with or without the addition of protease inhibitors. The results showed that despite the absence of protease inhibitors the expression of the 230 KD antigen was not affected, which indicated that the 160 KD protein is not a breakdown product of the major 230 KD antigen. However, proteolysis caused the degradation of the 160 KD antigen to a 140 KD fragment.

To determine the relationship of different antigens detected by Western immunoblotting, Mayer et al.[251] affinity purified the bullous pemphigoid antibodies on the different antigens. The eluted antibodies were subsequently used in immunoblotting assays. The antibodies affinity purified from the 240 KD and the 160 KD band stained the corresponding antigens on nitrocellulose blots with no cross reactivity. Although this result confirmed that the 160 KD antigen is not a degradation product of the 230 KD antigen, it remains possible that it is a proteolytic fragment of a 180 KD antigen[251]. In a similar study Robledo et al.[252] detected the 230 KD and a 180 KD antigen by Western immunoblotting and re-examined eluted IgG antibodies from these bands. Binding of affinity purified antibodies to the corresponding antigens occurred without cross reactivity. Their result provided evidence that the two antigens are distinct and that the 180 KD antigen is not a breakdown product of the 220-240 KD antigens.

As in bullous pemphigoid, Western immunoblotting has been used to investigate the antigen reactive with sublamina densa antibodies from patients with epidermolysis bullosa acquisita. The target antigen has been identified as a polypeptide consisting of two components with molecular weights of 290 KD and 145 KD[146]. The antigen is a specific type of collagen that is associated with anchoring fibrils[129]. Circulating anti-basement membrane

antibodies from patients with blistering disorders including bullous pemphigoid, dermatitis herpetiformis, herpes gestationis and pemphigus do not recognize this antigen[146,240].

In the present study a high titre epidermolysis bullosa acquisita serum, with dermal staining pattern on split skin, was tested by Western immunoblotting but showed no specific bands. The failure of some epidermolysis bullosa acquisita antibodies to react with antigen on immunoblots has been reported[240,253]. In a study by Stanley et al.[253] of 5 epidermolysis bullosa acquisita sera the 290 KD antigen was identified by 4 samples whereas one serum did not stain any bands. Conversely, by immunoprecipitation, which does not involve denaturing of proteins, the 290 KD antigen was precipitated by all 5 sera. The authors suggested that some epidermolysis bullosa acquisita sera contain antibodies which do not bind denatured antigen on immunoblots.

Although sera from some patients with bullous pemphigoid stain both the epidermis and dermis of split skin, studies have demonstrated that they do not contain antibodies against the epidermolysis bullosa antigen[240,254].

The results of different reports and the present study have shown that bullous pemphigoid sera always produce an epidermal or combined staining pattern when tested on split skin regardless of their antigenic specificity as determined by Western immunoblotting. In

addition, it has been confirmed that antibodies from patients with epidermolysis bullosa acquisita consistently bind to the dermal side of sodium chloride separated skin. Sera with such a staining pattern regularly recognize the 290 KD and 145 KD antigen.

These observations therefore indicate good correlation between the indirect immunofluorescence with split skin and Western immunoblotting findings and support the value of these techniques in establishing the correct diagnosis in bullous pemphigoid and acquired epidermolysis bullosa.

4.4 ANTI-ENDOMYSIAL ANTIBODIES IN DERMATITIS HERPETIFORMIS

Circulating IgA class antibodies to the lining of smooth muscle fibrils can be detected by indirect immunofluorescence using smooth muscle-containing substrate such as monkey oesophagus. The diagnostic significance of these antibodies was determined in the present study by testing 20 patients with dermatitis herpetiformis, 20 with pemphigus and 20 with bullous pemphigoid. The overall sensitivity of the anti-endomysial antibodies for the diagnosis of dermatitis herpetiformis was 60%. However, a higher frequency was observed in patients on a non-

restricted diet with normal gluten intake. The antibodies were detected in 11 (73%) of 15 patients who were on a normal diet. Conversely, the endomysial antibodies occurred in only one (20%) of the five patients following a gluten-free diet. The antibodies could not be detected in any of the control sera from patients with pemphigus or bullous pemphigoid.

The anti-endomysial antibodies were first described in 1983 by Chorzelski et al.[172]. Table 23 indicates the incidence of these antibodies in various studies. The antibodies, which are found in dermatitis herpetiformis and coeliac disease, are directed against the endomysium of smooth muscle fibres and are predominantly of the IgA class although IgG antibodies may be seen in low titres. The target of these antibodies appears to be the reticular component associated with the endomysium of smooth muscle. The antibodies, however, do not react with reticular fibrils of other organs[172]. Chorzelski et al.[255] performed indirect immunofluorescence tests on oesophagi of selected species to determine the most suitable for the reactivity of anti-endomysial antibodies. The antibodies did not react with the endomysium of the smooth muscle of mouse and rat and negative or low antibody levels were seen with oesophagus of cattle, calf, goat and guinea pig. The highest titres were obtained when monkey oesophagus was used as a substrate. These studies confirmed that the smooth muscle of monkey oesophagus is the most appropriate

Table 23

THE INCIDENCE OF ANTI-ENDOMYSIAL ANTIBODIES

Date	Study Group	No.* Tested	No. Positive	% Positive
1983	Chorzelski et al.(172)	38	26	68
1984	Chorzelski et al.(255)	38	30	79
1985	Leonard et al.(265)	12	8	67
1985	Meurer et al.(260)	7	6	86
1986	Katz et al.(259)	33	17	51
1987	Reunala et al.(263)	29	22	76
1987	Kumar et al.(272)	40	26	65
1988	Chorzelski et al.(266)	2	2	100
1989	Peters and McEvoy(264)	24	19	79
1992	Alkarawi	15	11	73

* Dermatitis herpetiformis cases on normal diet

tissue for this serologic test. However, differences in the distribution of the endomysium antigen at various levels of the oesophagus between the pharynx and the stomach have been reported[256]. The variability in the expression of this antigen is a consequence of the anatomic distribution of smooth and skeletal muscle between the upper and the lower oesophagus. The reactivity of sera containing anti-endomysial antibodies with monkey oesophagus would depend on the use of sections obtained from an appropriate site. Kumar et al.[256] studied the distribution of the endomysium antigen throughout the entire length of the oesophagus using serum from a patient with dermatitis herpetiformis. The immunofluorescence results showed that the antigen was completely absent in the upper oesophagus whereas sections obtained from the middle part of the oesophagus showed positive and negative muscle fibres. In contrast, the greatest amount of antigen was observed in the lower oesophagus.

The frequencies of IgA anti-endomysial antibodies in the original report of Chorzelski et al.[172] were 68% and 60% in dermatitis herpetiformis and coeliac disease respectively. The same group[255] confirmed these observations in a subsequent report and showed that the antibodies are not detectable in other skin disorders including pemphigus and bullous pemphigoid. Of particular interest was the finding of negative results with sera ob-

tained from patients with linear IgA bullous dermatosis which emphasized that this disorder is distinct from dermatitis herpetiformis.

On the initial studies on endomysial antibodies some false positive results were reported after testing sera from patients with gut diseases other than coeliac disease, such as malabsorption syndrome and milk-sensitive enteropathy. However, follow-up and re-examination of these cases showed that circulating anti-endomysial antibodies were only detected in patients subsequently proved to have coeliac disease[255,257]. Further studies of patients with other enteropathies including ulcerative colitis and Crohn's disease confirmed that the endomysial antibodies are specific of gluten enteropathy[257].

Chorzelski et al.[255] reported an association between the presence as well as the titre of anti-endomysial antibodies and the severity of intestinal pathology. they demonstrated a correlation between these antibodies and the extent of mucosal damage in the gut. Almost all patients with jejeunal mucosal atrophy of grades III and IV had the endomysium antibodies.

Despite the fact that dermatitis herpetiformis is associated with a milder degree of gluten-sensitive enteropathy than coeliac disease[258], the endomysial antibodies were detected in a higher proportion in the former disease[172,255]. Subsequent studies, however, showed that the endomysial antibodies were found in 100%

of coeliac disease patients when they are tested prior to the institution of gluten-free diet which is an essential part of the management in this disorder[259]. The effect of gluten-free diet on endomysial antibodies in dermatitis herpetiformis has also been studied. Muerer et al.[260] investigated six seropositive patients with a pathologically confirmed enteropathy. Treatment of this group of patients with gluten-free diet was associated with the progressive decline then the disappearance of endomysial antibodies within 4-11 months. In contrast, the antibody titres are not affected by the treatment with dapsone or sulfapyridine without dietary therapy[260,261]. A similar effect of gluten-free diet on the levels of endomysium antibodies in childhood cases of dermatitis herpetiformis has also been described. Chorzelski et al.[262] studied 32 patients with this diagnosis who had been following a gluten-free diet and showed that the presence of endomysium antibodies is related to the duration and adherence to the diet. The antibodies were detected in one patient before treatment but were absent after one month of dietary therapy. The endomysial antibodies remained detectable in children treated for less than one year, and in the majority of patients not strictly adhering to the gluten-free diet. These findings were also confirmed and extended by other investigators. In a study of 29 dermatitis herpetiformis patients Reunala et al.[263] showed that endomysial antibodies correlate with the

severity of jejeunal atrophy. The mucosal damage was graded as subtotal villous atrophy, partial villous atrophy or slight changes-normal mucosa. The anti-endomysial antibodies were detected in all patients with subtotal villous atrophy and in the majority of patients with partial villous atrophy. Conversely, the lowest frequency and antibody levels were found in patients with slight changes or normal mucosa. Based on this observation, it is possible that seronegative patients on normal diet found in the present study and previous investigations[257,259,264] might have had only minimal or no jejeunal mucosal atrophy. Reunala et al.[263] placed some patients on a gluten-free diet and performed sequential antibody titre estimations. The treatment was associated with a decline in the endomysial antibody levels in patients who adhered to the gluten-free diet for a few months. In contrast, there was no fall in antibody titres in a group of patients on a normal diet. Furthermore, one initially seronegative patients developed endomysial antibodies while continuing on normal gluten-containing diet, which may have caused some damage to the jejeunal mucosa.

Leonard et al.[265] further investigated the association between gluten-sensitive enteropathy and endomysial antibodies. They studied 12 dermatitis herpetiformis patients who were controlled by strict gluten-free diet and challenged with gluten. Prior to challenge, jejeunal

biopsies showed essentially normal mucosa and serologic tests for endomysial antibodies were negative in all patients. The ingestion of gluten was followed by the appearance of antibodies and deterioration of intestinal mucosa (graded on a scale of I-IV) in a proportion of patients. In six cases the rise of endomysial antibody titre correlated with a deterioration of gluten enteropathy. In one patient who showed changes in gut histopathology but did not become seropositive, the mucosal damage was only of grade I-II. Importantly, the study included evidence of the reappearance and rise of endomysial antibodies occurring prior to the development of pathological changes of mucosa.

The effect of gluten challenge on endomysial antibodies and the morphology of the intestinal mucosa was also determined by Chorzelski et al.[266]. They studied two dermatitis herpetiformis patients with normal jejeunal mucosa and negative serology for endomysial antibodies. Aggressive gluten challenge for 3-5 months was associated with the appearance of endomysial antibodies. In addition, pathological examination of jejeunal mucosa in one patient showed changes of grade III. Treatment with gluten-free diet led to the decline of endomysial antibodies to undetectable levels and the return of gut morphology to grade II.

The high degree of sensitivity and specificity of circulating anti-endomysial antibodies in patients with dermatitis herpetiformis has led to the suggestion that they may have a pathogenic significance[257]. To examine whether the antibodies are of a pathogenic nature, in vitro studies were performed to determine if the endomysial antibodies are capable of reacting with their homologous antigen in explants of normal gut mucosa. Indirect immunofluorescence studies of endomysial antibodies on sections of normal monkey gut mucosa explant cultured in human serum showed a positive reaction. However, no reaction was observed by direct immunofluorescence using sections from the normal gut explants which indicated that the endomysial antibodies cannot react with their homologous antigen in viable explants. In contrast, positive direct immunofluorescence reactions were obtained with pemphigus and bullous pemphigoid antibodies under comparable conditions[257].

Despite these data, the detectibility of endomysial antibodies after gluten challenge and their appearance prior to the deterioration of gut pathology suggests that they may be pathogenic antibodies.

In addition to IgA anti-endomysial antibodies, other antibodies including anti-gliadin and anti-reticulin antibodies have been reported in patients with dermatitis herpetiformis. Anti-gliadin antibodies of the IgG class have been detected in the majority of patients[267].

However, these antibodies lack disease specificity as they have been demonstrated in normal individuals and patients with other bullous diseases[267,268]. Conversely, IgA class anti-gliadin antibodies are more disease specific but occur in only a small proportion of dermatitis herpetiformis patients[267,269]. Anti-reticulin antibodies may also be of the IgG or IgA class. IgG anti-reticulin antibodies occur in 20% of patients and, like IgG gliadin antibodies, are not specific for dermatitis herpetiformis[270]. In contrast, anti-reticulin antibodies of the IgA class are specific for dermatitis herpetiformis and coeliac disease[271]. Hallstrom[271] studied the occurrence of IgA reticulin and endomysial antibodies in patients with dermatitis herpetiformis. They found the frequencies of both antibodies to be similar. In another report Kumar et al.[272] compared the incidence of these antibodies in 45 dermatitis herpetiformis patients and in a control group of normal individuals and patients with pemphigus and bullous pemphigoid. The endomysial antibodies were detected in about two-thirds of dermatitis herpetiformis patients on normal diet but in none of the patients on gluten free diet or the control group. IgG anti-reticulin antibodies were observed in one-third of dermatitis herpetiformis patients and were also detected in the control group including healthy individuals.

In contrast, anti-reticulin antibodies of the IgA class were disease specific but occurred only in 25% of dermatitis herpetiformis patients. Furthermore, these antibodies were not detected in any of the sera that were negative for the endomysial antibodies. These results provide evidence of the diagnostic importance of immunofluorescence tests for endomysial antibodies. The endomysial antibody test has been used in evaluating suspected cases of dermatitis herpetiformis with equivocal histologic and direct immunofluorescence findings. Studies have suggested that patients positive for IgA endomysial antibodies but with negative direct immunofluorescence for IgA deposits may eventually prove to have dermatitis herpetiformis[261,273]. Accetto et al.[261] reported three such cases with atypical clinical features. The initial biopsy specimens yielded negative or non-diagnostic histologic and direct immunofluorescence features. However, serologic studies showed high titres of endomysial antibodies. Immunofluorescence studies of subsequent skin biopsies demonstrated IgA deposits in all patients.

In summary, the results of the present study and previous investigations indicate the high degree of specificity and sensitivity of the endomysial antibodies for the diagnosis of dermatitis herpetiformis and emphasize the value of this serologic test in the study of patients with bullous disorders. The availability of the

test is of particular importance in confirming the clinical diagnosis of dermatitis herpetiformis cases where the investigations may be negative or non-specific. In addition, the demonstration of endomysial antibodies is a useful screening test to determine the presence of associated gluten enteropathy. The absence of the antibodies in patients following a gluten-free diet indicates that the endomysial antibody assay is a helpful, minimally invasive method to monitor the compliance of patients to dietary therapy and decrease the need for repeated intestinal biopsies.

5. CONCLUSIONS

The present study has demonstrated quantitative differences in the expression of bullous pemphigoid antigen on normal skin that appear to be related to the body site from which a particular skin specimen is obtained. There was only partial correlation between antigen expression and the distribution of lesions in bullous pemphigoid. Conversely, the amount of pemphigus vulgaris antigen was generally similar in the areas tested. These findings indicate that the distribution of lesions in these disorders is not entirely dependent on the concentration of antigen in a particular area.

Indirect immunofluorescence testing of 36 serum samples from patients with pemphigoid and pemphigus produced positive staining reactions on three different epithelial substrates but showed variable antibody levels. These results indicated that the choice of epithelial source may influence antibody titres. This, however, does not represent a major problem in evaluating the indirect immunofluorescence test because the variations related to the type of substrate used do not affect the detectability of pemphigoid and pemphigus antibodies.

Examination of the distribution of IgG subclasses in the skin and serum of bullous pemphigoid patients showed a predominance of the non-complement fixing IgG_4. Studies of the capability of serum antibodies to fix complement revealed non-complement fixing sera and the results were not in agreement with the complement binding characteristics of IgG subclasses. These findings argue against a primary role for complement in the induction of bullous pemphigoid and since IgG_4 has homocytotropic properties for mast cells then this favours the view that it may play a role in mast cell degranulation with subsequent inflammation and blister formation.

IgG_4 was also the prevalent IgG subclass in the skin and serum of patients with pemphigus vulgaris. Although there is no evidence that IgG_4 is pathogenic in this disorder, the clinical and immunological reproduction of endemic pemphigus foliaceus in neonatal mice injected with IgG_4 antibodies obtained from patients affected with this form of pemphigus suggests that the subclass may also be involved in the pathogenesis of pemphigus vulgaris.

It was concluded from the immunofluorescence testing of sera containing anti-basement membrane zone antibodies on 1M sodium chloride-separated skin that bullous pemphigoid and epidermolysis bullosa acquisita antibodies produce two different staining patterns on this substrate. Furthermore, it was demonstrated that the same technique can be applied to patients' skin to determine the site of

in vivo bound immune deposits, which is particularly useful in patients with no detectable circulating antibodies. In view of the clinical, histologic and immunopathologic overlap between bullous pemphigoid and epidermolysis bullosa acquisita, the definite diagnosis of some cases could previously be determined only by immunoelectron microscopy or immunochemical methods. However, the development of the split skin technique now provides a relatively simple yet efficient method that can be used to reach a certain diagnosis without the need to resort to other sophisticated laboratory techniques.

Western immunoblotting studies of pemphigoid sera showed that circulating anti-basement membrane zone antibodies react with specific protein bands on immunoblots and demonstrated heterogeneity of the bullous pemphigoid antigen at the molecular level.

The present study confirmed that endomysial antibodies are frequently detected in dermatitis herpetiformis patients on a non-restricted diet but are absent in the majority of patients following a gluten-free diet. The antibodies were also not detectable in the sera of patients with other bullous disorders. These results indicate that the demonstration of anti-endomysial antibodies is of considerable value in the diagnosis of dermatitis herpetiformis and the assessment of patients with bullous diseases with clinical and histologic features that may simulate those of dermatitis herpetiformis. In

addition, the endomysial antibody test is a simple and useful method to verify patients' adherence to a gluten-free diet and decrease the requirement for repeated intestinal biopsies.

REFERENCES

1. Lever WF. Pemphigus. Medicine 1953; 32: 1-123.

2. Lever WF. Pemphigus and Pemphigoid. Springfield, IL: Charles C Thomas. 1965.

3. Bean SF, Michel B, Furey N. Vesicular pemphigoid. Arch Dermatol 1976; 112: 1402-4.

4. Goldberg DJ, Sabolinski M, Bystryn JC. Regional variation in the expression of bullous pemphigoid antigen and location of lesions in bullous pemphigoid. J Invest Dermatol 1984; 82: 326-28.

5. Person JR, Rogers RS. Bullous and cicatricial pemphigoid. Mayo Clinic Proc 1977; 52: 54-66.

6. Ahmed AR, Maize JC, Provost TT. Bullous pemphigoid: clinical and immunologic follow-up after successful therapy. Arch Dermatol 1977; 113: 1043-46.

7. Hodge L, Marsden RA, Black MM, Bhogal B, Corbett MF. Bullous pemphigoid: the frequency of mucosal involvement and concurrent malignancy related to indirect immunofluorescence findings. Br J Dermatol 1981; 105: 65-69.

8. Barker DJ. Generalized pruritus as the presenting feature of bullous pemphigoid. Br J Dermatol 1983; 109: 237-38.

9. Bingham EA, Burrows D, Sandford JC. Prolonged pruritus and bullous pemphigoid. Clin Exp Dermatol 1984; 9: 564-70.

10. Chaung TY, Korkij W, Soltani K et al. Increased frequency of diabetes mellitus in patients with bullous pemphigoid: A case control study. J Am Acad Dermatol 1984; 11: 1099-1102.

11. Koorbor WA, Price NM, Watson W. Coexistent psoriasis and bullous pemphigoid. Arch Dermatol 1978; 114: 1643-46.

12. Robinson JK, Baughman RD, Provost TT. Bullous pemphigoid induced by PUVA therapy. Is this the etiology of the acral bullae produced during PUVA treatment? Br J Dermatol 1978; 99: 709-13.

13. Grattan CEH. Evidence of an association between bullous pemphigoid and psoriasis. Br J Dermatol 1985; 113: 281-83.

14. Giannini JM, Callen JP, Gruber GG. Bullous pemphigoid and rheumatoid arthritis. J Am Acad Dermatol 1981; 4: 695-97.

15. Stone SP, Schroeter AL. Bullous pemphigoid and associated malignant neoplasms. Arch Dermatol 1985; 111: 991-94.

16. Rook AJ. A pemphigoid eruption associated with carcinoma of the bronchus. Trans. St. John's Hosp Dermatol Soc 1968; 54: 152-55.

17. Ahmed AR, Chu TM, Provost TT. Bullous pemphigoid. Clinical and serologic evaluation for associated malignant neoplasms. Arch Dermatol 1977; 113: 969.

18. Lever WF. Pemphigus and pemphigoid. A review of the advances made since 1964. J Am Acad Dermatol 1979; 1: 2-31.

19. Stevenson CJ. Treatment in bullous disease with corticosteroid drugs and corticotrophin. Br J Dermatol 1960; 72: 11-21.

20. Downham JF, Chapel TA. Bullous pemphigoid: therapy in patients with and without diabetes mellitus. Arch Dermatol 1978; 114: 1639-42.

21. Person JR, Rogers RS, Perry HO. Localized pemphigoid. Br J Dermatol 1976; 95: 531-34.

22. Provost TT, Maize JC, Ahmed AR, Strauss JS, Dobson RL. Unusual subepidermal bullous disease with immunologic features of bullous pemphigoid. Arch Dermatol 1979; 115: 156-60.

23. Robinson JW, Odom RB. Bullous pemphigoid in children. Arch Dermatol 1978; 114: 899-902.

24. Gruber GG, Owen LG, Callen JP. Vesicular pemphigoid. J Am Acad Dermatol 1980; 3: 619-22.

25. Honeyman JF, Honeyman AR, De la Parra MA, Pinto A, Eguiguren G. Polymorphic pemphigoid. Arch Dermatol 1979; 115: 423-27.

26. Winkelmann RK, Daniel Su WP. Pemphigoid vegetans. Arch Dermatol 1979; 115: 446-48.

27. Massa C, Connelly SM. Bullous pemphigoid with features of prurigo nodularis. Arch Dermatol 1982; 118: 937-39.

28. Roenigk H, Ryan JG, Bergfeld WF. Epidermolysis bullosa acquisita. Arch Dermatol 1971; 103: 1-10.

29. Gammon WR, Briggaman RA, Wheeler CE. Epidermolysis bullosa acquisita presenting as an inflammatory bullous disease. J Am Acad Dermatol 1982; 7: 382-87.

30. Katz SI. Dermatitis herpetiformis. In: Fitzpatrick TB, Eisen AZ, Wolff K et al. (eds). Dermatology in General Medicine. New York: McGraw Hill. 1987. Pp.593-97.

31. Lever WF, Schaumburg-Lever G. Histopathology of the Skin, 6th ed.. Philadelphia: JB Lippincott. 1983.

32. Leonard JN, Haffenden GP, Ring NP et al. Linear IgA disease in adults. Br J Dermatol 1982; 107: 301-16.

33. Hall RP, Sanders ME, Duquesnoy RJ et al. Alterations in HLA-DP and HLA-DQ antigen frequency in patients with dermatitis herpetiformis. J Invest Dermatol 1989; 93: 501-5.

34. Hardy KM, Perry HO, Pingree GC, Kirby TJ Jr. Benign mucous membrane pemphigoid. Arch Dermatol 1971; 104: 467-75.

35. Church RE, Sneddon IB. Ocular pemphigus with generalized bullous eruption. Br J Dermatol 1956; 68: 128-31.

36. Brunsting LA, Perry HO. Benign pemphigoid? A report of seven cases with chronic, scarring, herpetiform plaques about the head and neck. Arch Dermatol 1957; 75: 489-501.

37. Lever WF, Hashimoto K. The etiology and treatment of pemphigus and pemphigoid. J Invest Dermatol 1969; 53: 373-89.

38. Kobayasi T. The dermo-epidermal junction in bullous pemphigoid. Dermatologica 1967; 134: 157-65.

39. Schaumberg-Lever G, Orfanos CE, Lever WF. Electron microscopic study of bullous pemphigoid. Arch Dermatol 1972; 106: 662-67.

40. Wagner V, Seba G, Hruskova B. Demonstration of tissue autoantibodies in the blood serum of patients with certain skin diseases. Dermatologica 1956; 112: 25-36.

41. Beutner EH, Jordon RE. Demonstration of skin antibodies in sera of pemphigus vulgaris patients by indirect immunofluorescence staining. Proc Soc Exp Biol Med 1964; 117: 505-10.

42. Beutner EG, Lever WF, Witebsky E et al. Autoantibodies in pemphigus vulgaris. Response to an intercellular substance of epidermis. JAMA 1965; 192: 682-88.

43. Chorzelski TP, Jablonska S, Blaszczyk M. Autoantibodies in pemphigus. Acta Dermato-vener 1966; 47: 26-33.

44. Jordon RF, Beutner EH, Witebsky E et al. Basement membrane zone antibodies in bullous pemphigoid. JAMA 1967; 200: 751-56.

45. Beutner EH, Rhodes EL, Holborow EJ. Autoimmunity in chronic bullous skin diseases. Clin Exp Immun 1967; 2: 141-51.

46. Beutner EG, Jordon RE, Chorzelski TP. The immunopathology of pemphigus and bullous pemphigoid. J Invest Dermatol 1968; 51: 63-80.

47. Chorzelski TP, Jablonska S, Blaszczyk M. Autoantibodies in pemphigoid. Dermatologica 1968; 136: 325-34.

48. Gammon WR. The immunopathology of bullous pemphigoid antibodies. In: Beutner EH, Chorzelski TP, Kumar V (eds). Immunopathology of the Skin, 3rd ed. New York: Wiley and Sons. 1987. Pp.323-36.

49. Carlo JR, Gammon WR, Sams WM, Ruddy S. Demonstration of the complement regulating protein, B1H, in skin biopsies from patients with bullous pemphigoid. J Invest Dermatol 1979; 73: 551-53.

50. Provost TT, Tomasi TB Jr. Immunopathology of bullous pemphigoid. Clin Exp Immunol 1974; 18: 193-200.

51. Weigand D. Effect of anatomic region on immunofluorescence diagnosis of bullous pemphigoid. J Am Acad Dermatol 1985; 12: 274-78.

52. Jordon RE, Heine KG, Tappanier C et al. The immunopathology of herpes gestationis. Immunofluorescence studies and characterization of "HG factor". J Clin Invest 1976; 57: 1426.

53. Kushniruk W. The immunopathology of epidermolysis bullosa acquisita. Can Med J 1973; 108: 1143-46.

54. Olansky AJ, Briggaman RA, Gammon WR et al. Bullous systemic lupus erythematosus. J Am Acad Dermatol 1982; 7: 511-20.

55. Holubar K, Wolff K, Konrad K et al. Ultrastructural localization of immunoglobulins in bullous pemphigoid. J Invest Dermatol 1975; 64: 220-27.

56. Niebuer C, Boorsma DM, Woerderman MJ, Klasbeek GL. Epidermolysis bullosa acquisita. Br J Dermatol 1980; 102: 383-92.

57. Tuffanelli DL. Cutaneous immunopathology. Recent observations. J Invest Dermatol 1975; 65: 143-53.

58. Kumar V, Binder WL, Schotland E et al. Coexistence of bullous pemphigoid and systemic lupus erythematosus. Arch Dermatol 1978; 114: 1187-90.

59. Herrmann K, Lohrisch I, Böhme HJ et al. Detection of antibodies after immune complex splitting in serum of patients with bullous pemphigoid and systemic lupus erythematosus. Br J Dermatol 1978; 99: 635-40.

60. Millns JL, Meurer M, Jordon RE. The complement system in bullous pemphigoid. VI. C_3 fixing activity in the absence of detectable antibody. Clin Immunol Immunopath 1979; 13: 475-83.

61. Chorzelski TP, Beutner EH. Factors contributing to occasional failures in the laboratory diagnosis of bullous pemphigoid by indirect immunofluorescence. Br J Dermatol 1972; 86: 111-17.

62. Bystryn JC, Sabolinski M. Effect of substrate on indirect immunofluorescence tests for intercellular and basement membrane zone antibodies. J Am Acad Dermatol 1986; 15: 973-77.

63. Peck SM, Osserman KE, Lefkovits A, Osserman BS. Studies in bullous diseases: immunofluorescent serologic tests. New Eng J Med 1968; 279: 951-58.

64. Katz S, Halprin KM, Inderbitzin TM. The use of human skin for the detection of antiepithelial autoantibodies. J Invest Dermatol 1969; 53: 390-99.

65. Sams WM, Jordon RE. Correlation of pemphigoid and pemphigus antibody with activity of disease. Br J Dermatol 1971; 84: 7-13.

66. Maize JC, Provost TT. Value of immunofluorescent techniques in studies of bullous disease. Am J Dermatopath 1983; 5: 67-72.

67. Korman N. Pemphigus. J Am Acad Dermatol 1988; 18: 1219-38.

68. Meurer M, Millns JL, Rogers RS III, Jordon RE. Oral pemphigus vulgaris. A report of ten cases. Arch Dermatol 1977; 113: 1520-24.

69. Judd KP, Lever WF. Correlation of antibodies in skin and serum with disease severity in pemphigus. Arch Dermatol 1979; 115: 428-32.

70. Beutner EH, Chorzelski TP, Jordon RE. Autosensitization in pemphigus and bullous pemphigoid. Springfield, IL: Charles C Thomas. 1970. Pp.69-81.

71. Whittingham, S, Mackay IR. The "pemphigus" antibody and immunopathies affecting the thymus. Br J Dermatol 1971; 84: 1-6.

72. Quismorio FP, Bland SL, Friou GJ. Autoimmunity in thermal burns: occurrence of rheumatoid factors, antinuclear antibodies and antiepithelial antibodies. Clin Exp Immunol 1971; 8: 701-11.

73. Acosta E, Ivanyi L. Comparison of the reactivity of various epithelial substrates for the titration of pemphigus antibodies by indirect immunofluorescence. Br J Dermatol 1982; 107: 537-41.

74. Kransy SA, Beutner EH, Chorzelski TP. Specificity and sensitivity of indirect and direct immunofluorescent findings in the diagnosis of pemphigus. In: Beutner EG, Chorzelski TP, Kumar V (eds). Immunopathology of the Skin, 3rd ed. New York: Wiley and Sons. 1987. Pp.207-47.

75. Chorzelski TP, Von Weiss J, Lever WF. Clinical significance of autoantibodies in pemphigus. Arch Dermatol 1966; 93: 570-76.

76. Weissman V, Feuerman EJ, Joshua H, Hazaz B. The correlation between the antibody titres in sera of patients with pemphigus vulgaris and their clinical state. J Invest Dermatol 1978; 71: 107-09.

77. O'Loughlin S, Goldman GC, Provost TT. Fate of pemphigus antibody following successful therapy. Arch Dermatol 1978; 17: 69-72.

78. Fitzpatrick RE, Newcomer VD. The correlation of disease activity and antibody titres in pemphigus. Arch Dermatol 1980; 116: 285-90.

79. Creswell SN, Black MM, Bhogal B et al. Correlation of circulating intercellular antibody titres in pemphigus with disease activity. Clin Exp Dermatol 1981; 6: 477-83.

80. Anderson HJ, Newcomer VD, Landau JW et al. Pemphigus and other diseases. Arch Dermatol 1970; 101: 538-46.

81. Dray S. Three gamma-globulins in normal skin revealed by monkey precipitins. Science 1960; 132: 1313-14.

82. Grey HM, Kunkel HG. H chain subgroups of myeloma proteins and normal 7S gammaglobulin. J Exp Med 1964; 120: 253-66.

83. Terry WD, Fahey JL. Subclasses of human gamma 2-globulin based on differences in the heavy polypeptide chains. Science 1964; 146: 400-01.

84. Saluk PH, Clem LW. The unique molecular weight of the heavy chain from human IgG_3. J Immunol 1971; 107: 298-301.

85. Frangione B, Milstein C, Pink JRL. Structural studies of immunoglobulins. Nature 1969; 221: 145-48.

86. Grey HM, Kohler PF, Terry WD, Franklin EC. Human monoclonal gamma cryoglobulins with anti-gamma globulin activity. J Clin Invest 1968; 47: 1875-84.

87. Jefferis R, Weston PD, Stanworth DR. Relationship between the papain sensitivity of human gamma immunoglobulins and the heavy chain subclasses. Nature 1968: 219: 646.

88. Turner MW, Bennich HH, Notvig JB. Pepsin digestion of human IgG myeloma proteins of different subclasses. I. The characteristic feature of pepsin cleavage as a function of time. Clin Exp Immunol 1970; 7: 603-25.

89. Stanworth DR. Immunochemical aspects of IgG_4. Clin Rev Allergy 1983; 1: 183-95.

90. Ishizaka T, Ishizaka K, Salmon S, Fudenberg HH. Biologic activities of aggregated gamma globulin. VIII. Aggregated immunoglobulin of different classes. J Immunol 1967; 99: 82-91.

91. Schumaker VN, Calcott MA, Spiegelberg HL, Müller-Eberhard HJ. Ultracentrifuge studies of the binding of IgG of different subclasses to the C_{1q} subunit of the first component of complement. Biochem 1976; 15: 5175-81.

92. Hautanen A, Sernesto A, Keski-Oja J et al. C_{3c} and C_{3d} fragments of human C_3 bind myeloma IgG_1 and IgG_3 proteins. Immunol 1982; 5: 227-32.

93. Morell A, Terry WD, Waldmann TA. Metabolic properties of IgG subclasses in man. J Clin Invest 1970; 49: 673-80.

94. Spiegelberg HL, Fishkin BG, Grey HM. Catabolism of G-myeloma proteins in man. J Clin Invest 1968; 47: 2323-30.

95. Franklin EC, Frangione B. Common structural and antigenic properties of human anti-gamma globulins. J Immunol 1971; 107: 1527-34.

96. Simmons JG, Fuller CR, Bulchonan PD et al. Distribution of surface, cytoplasmic and secreted IgG subclasses in human lymphoblastoid cell lines and normal peripheral blood lymphocytes. Scand J Immunol 1981; 14: 1-13.

97. Heiner D. Significance of immunoglobulin G subclasses. Am J Med 1984; 76(Suppl.): 1-6.

98. Endresen GK, Forre O. Studies on the binding of immunoglobulins and immune complexes to the surface of human platelets: IgG molecules react with platelet Fc receptors with the CH3 domain. Int Arch Allergy Appl Immunol 1982; 67: 33-39.

99. Von dem Borne AE, Van Leeuwen EF, Von Riesz LE, Van Boxtel CJ, Engelfriet CP. Neonatal alloimmune thrombocytopenia: detection and characterization of the responsible antibodies by the platelet immunofluorescent test. Blood 1981; 57: 649-56.

100. Huber H, Douglas SD, Nusbacher J, Kochwa S, Rosenfeld RE. IgG subclass specificity of human monocyte receptor sites. Nature 1971; 229: 419-20.

101. Messner RP, Jelinck J. Receptors for human G globulin on human neutrophils. J Clin Invest 1970; 49: 2165-71.

102. Nakegawa T, de Weck AL. Membrane receptors for the gG subclass on human basophils and mast cells. Clin Rev Allergy 1983; 1: 197-207.

103. Vijay HM, Perelmutter L. Inhibition of reagin-mediated PCA reactions in monkeys and histamine release from human leucocytes by human IgG_4 subclass. Int Arch Allergy Appl Immunol 1977; 53: 78-87.

104. Van Toorenenbergen AW, Aalberse RC. IgG_4 and passive sensitization of basophil leucocytes. Int Arch Allergy Appl Immunol 1981; 65: 432-40.

105. Devey ME, Wilson DV, Wheeler AW. The IgG subclasses of antibodies to grass pollen allergens produced in hay fever patients during hyposensitization. Clin Allergy 1976; 6: 227-36.

106. Van der Giessen M, Homan WL, van Kernebeek G et al. Subclass typing of IgG antibodies formed by grass pollen allergic patients during immunotherapy. Int Arch Allergy Appl Immunol 1976; 50: 625-39.

107. Jones CC, Hamilton RG, Jordon RE. Subclass distribution of human IgG autoantibodies in pemphigus. J Clin Immunol 1980; 8: 43-49.

108. Bird P, Friedmann PS, Ling N et al. Subclass distribution of IgG autoantibodies in bullous pemphigoid. J Invest Dermatol 1986; 86: 21-25.

109. Kelly SE, Cerio R, Bhogal BS, Black MM. The distribution of IgG subclasses in pemphigoid gestationis: PG factor is an IgG_1 autoantibody. J Invest Dermatol 1989; 92: 695-98.

110. Eisenberg RA, Dyer K, Craven SY et al. Subclass restriction and polyclonility of the systemic lupus erythematosus marker antibody anti-Sm. J Clin Invest 1985; 75: 1270-77.

111. Schur PH, Monroe M, Rothfield N. The gamma G subclass of antinuclear and antinucleic acid antibodies. Arth Rheum 1972; 15: 174-82.

112. Persson MA, Brown SE, Steward MW et al. IgG subclass-associated affinity differences of specific antibodies in humans. J Immunol 1988; 140: 3875-79.

113. Young WJ, Dorner MM, Kunkel HG, Kabat EA. Studies on human antibodies. VI. Selective variations in subgroup composition and genetic markers. J Exp Med 1968; 127: 633-46.

114. Schur PH. Human gamma G subclasses. Prog Clin Immunol 1972; 1: 71-104.

115. Oxelius V. Quantitative and qualitative investigations of serum IgG subclasses in immunodeficiency diseases. Clin Exp Immunol 1979; 36: 112-16.

116. Vanlogheem E, Sukernik RI, Osipova LP et al. Gene deletion and gene duplication within the cluster of human heavy-chain genes. J Immunogenet 1980; 7: 285-99.

117. Oxelius VA. Immunoglobulin G (IgG) subclasses and human disease. Am J Med 1984; 76(Suppl.): 7-18.

118. Ambrosino DM, Schiffman G, Gotschlich EC et al. Correlation between $G_2m(n)$ immunoglobulin allotype and human antibody response and susceptibility to polysaccharide encapsulated bacteria. J Clin Invest 1985; 75: 1935-42.

119. LeFranc G, Dumitresco SM, Salier JP et al. Familial lack of the IgG_3 subclass. Gene elimination or burning off expression and neutral evolution in the immune system. J Immunogenet 1979; 6: 215-21.

120. Shakib F, McLaughlan P, Stanworth DR, Smith E, Fairburn E. Elevated serum IgE and IgG_4 in patients with atopic dermatitis. Br J Dermatol 1977; 97:59-63.

121. Gwynn CM, Smith JM, Leon GL, Stanworth DR. Role of IgG_4 subclass in childhood allergy. Lancet 1978; i: 910-11.

122. Parish WE. The clinical relevance of heat stable short term sensitizing anaphylactic IgG antibodies (IgG-S-TS) and of related activities of IgG_4 and IgG_2. Br J Dermatol 1981;105: 223-231.

123. Rock B, Martins CR, Theofilopoulos AN et al. The pathogenic effect of IgG_4 autoantibodies in endemic pemphigus foliaceus (Fogo selvagem). N Engl J Med 1989; 320: 1463-69.

124. Yaoita H, Briggaman RA, Lawley TJ, Katz SI. Epidermolysis bullosa acquisita: ultrastructural and immunological studies. J Invest Dermatol 1981; 76: 288-92.

125. Briggaman RA, Wheeler CE Jr. The epidermal-dermal junction. J Invest Dermatol 1975; 65: 71-84.

126. Foidart J-M, Bere E, Yaar M et al. Distribution and immunoelectron microscopic localization of laminin, a non collagenous basement membrane glycoprotein. Lab Invest 1980; 42: 336-42.

127. Yaoita H, Foidart J-M, Katz SI. Localization of the collagenous component in skin basement membrane. J Invest Dermatol 1978; 70: 191-93.

128. Bentz H, Morris N, Murray L. Isolation and partial characterization of a new human collagen with an extended triple-helical structural domain. Proc Natl Acad Sci 1983; 80: 3168-72.

129. Sakai Y, Keene DR, Morris NP et al. Type VII collagen is a major structural component of anchoring fibrils. J Cell Biol 1986; 103: 1577-86.

130. Stanley JR, Hawley-Nelson P, Poirier M, Katz SI, Yuspa SH. Detection of pemphigoid antigen, pemphigus antigen, and keratin filaments by indirect immunofluorescence in cultured human epidermal cells. J Invest Dermatol 1980; 75: 183-86.

131. Stanley JR, Hawley-Nelson P, Yuspa SH et al. Characterization of bullous pemphigoid antigen: a unique basement membrane protein of stratified squamous epithelia. Cell 1981; 24: 897-903.

132. Schaumburg-Lever G, Rule A, Schmidt-Ullrich B, Lever WF. Ultrastructural localization of in vivo bound immunoglobulins in bullous pemphigoid - a preliminary report. J Invest Dermatol 1975; 64: 47-49.

133. Pehamberger H, Gschnait F, Konrad K et al. Bullous pemphigoid, herpes gestationis and linear dermatitis herpetiformis: circulating anti-basement membrane zone antibodies; in vitro studies. J Invest Dermatol 1980; 74: 105-08.

134. Mutasim DF, Takahashi Y, Labib RS et al. A pool of bullous pemphigoid antigen(s) is intracellular and associated with the basal cell cytoskeleton-hemidesmosome complex. J Invest Dermatol 1985; 84: 47-53.

135. Westgate GE, Weaver AC, Couchman JR. Bullous pemphigoid antigen localization suggests an intracellular association with hemidesmosomes. J Invest Dermatol 1985; 84: 218-24.

136. Bridgen WD, Amos HE. The differential binding of antibody from the sera of patients with pemphigus and pemphigoid to isolated guinea pig epidermal cells. Br J Dermatol 1975; 93: 425-30.

137. Grekin PM, Levy GN, King AJ, Diaz LA. Some biochemical properties of pemphigoid antigen bound to the surface of dissociated epidermal basal cells. J Invest Dermatol 1981; 76: 190-192.

138. Regnier M, Vaigot P, Michel S et al. Localization of bullous pemphigoid antigen in isolated human keratinocytes. J Invest Dermatol 1985; 85: 187-90.

139. Mutasim DF, Morrison LH, Takahashi Y et al. Definition of bullous pemphigoid antibody binding to intracellular and extracellular antigen associated with hemidesmosomes. J Invest Dermatol 1989; 92: 225-30.

140. Diaz LA, Calvanico NJ, Tomasi TB, Jordon RE. Bullous pemphigoid antigen: isolation from normal human skin. J Immunol 1977; 118: 455-60.

141. Stanley JR, Hawley-Nelson P, Yaar M, Martin GR, Katz SI. Laminin and bullous pemphigoid antigen are distinct basement membrane proteins synthesized by epidermal cells. J Invest Dermatol 1982; 78: 456-59.

142. Stanley JR, Woodley DT, Katz SI. Identification and partial characterization of pemphigoid antigen extracted from normal human skin. J Invest Dermatol 1984; 82: 108-11.

143. Briggaman RA, Gammon WR, Woodley DT. Epidermolysis bullosa acquisita of the immunopathological type (dermolytic pemphigoid). J Invest Dermatol 1985; 85 (Suppl): 79-84.

144. Palestine RE, Kossard S, Dicken CH. Epidermolysis bullosa acquisita: a heterogenous disease. J Am Acad Dermatol 1981; 5: 43-53.

145. Dahl MGC. Epidermolysis bullosa acquisita: A sign of cicatricial pemphigoid? Br J. Dermatol 1979; 101: 475-83.

146. Woodley DT, Briggaman RA, O'Keefe EJ et al. Identification of the skin basement membrane autoantigen in epidermolysis bullosa acquisita. N Engl J Med 1984; 310: 1007-13.

147. Leigh IM, Eady RAJ, Heagarty AHM, Purkis PE, Whitehead PA, Burgeson RE. Type VII collagen is a normal component of basement membrane, which shows altered expression in recessive dystrophic epidermolysis bullosa. J Invest Dermatol 1988; 90: 639-42.

148. Yoshiika T, Woodley DT, Briggaman RA. Epidermolysis bullosa acquisita antigen: relationship between the collagenase-sensitive and insensitive domains. J Invest Dermatol 1988; 90: 127-33.

149. Paller AS, Queen LL, Woodley DT et al. A mouse monoclonal antibody against a newly discovered basement membrane component, the epidermolysis bullosa acquisita antigen. J Invest Dermatol 1985; 84: 215-17.

150. Stanley JR, Rubenstein N, Klaus-Kovtun V. Epidermolysis bullosa acquisita antigen is synthesized by both human keratinocyte and human dermal fibroblasts. J Invest Dermatol 1985; 85: 542-45.

151. Woodley DT, Burgeson RE, Linstrom G et al. The epidermolysis bullosa acquisita antigen is the globular carboxyl terminus of type VII procollagen. J Clin Invest 1988; 81: 683-87.

152. Duhring L. Dermatitis herpetiformis. JAMA 1884; 3: 225-28.

153. Pierard J, Whimster I. The histological diagnosis of dermatitis herpetiformis, bullous pemphigoid and erythema multiforme. Br J Dermatol 1961; 73: 253-66.

154. Costello M. Dermatitis herpetiformis treated with sulfapyridine. Arch Dermatol 1940; 41: 134.

155. Cormane RH. Immunofluorescent studies of the skin in lupus erythematosus and other diseases. Pathologia Eur 1967; 2: 170-80.

156. Pye RJ. Bullous eruptions. In: Rook A, Wilkinson DS, Ebling FJG, Champion RG, Burton JL (eds) Textbook of Dermatology, 4th ed. Oxford: Blackwell Scientific Publications. 1986. Pp.1619-63.

157. Campbell DG, Estes SA. A Koebner phenomenon in dermatitis herpetiformis. J Am Acad Dermatol 1983; 9: 767-68.

158. Marks JM. Dogma and dermatitis herpetiformis. Clin Exp Dermatol 1978; 2: 189-207.

159. Fry L, Leonard JN, Swain F et al. Long term follow-up of dermatitis herpetiformis with and without dietary gluten withdrawal. Br J Dermatol 1982; 107: 631-40.

160. Connor BL, Marks R, Wilson Jones E. Dermatitis herpetiformis. Trans St John's Hosp Dermatol Soc 1972; 58: 191-98.

161. Kint A, Geerts ML, De Brauwere D. Diagnostic criteria in dermatitis herpetiformis. Dermatologica 1976; 153: 266-71.

162. Eng AM, Moncada B. Bullous pemphigoid and dermatitis herpetiformis. Arch Dermatol 1974; 110: 51-57.

163. Van der Meer JB. Granular deposits of immunoglobulins in the skin of patients with dermatitis. An immunofluorescent study. Br J Dermatol 1969; 81: 493-503.

164. Seah PP, Fry L. Immunoglobulins in the skin in dermatitis herpetiformis and their relevance in diagnosis. Br J Dermatol 1975; 92: 157-66.

165. Fry L, Seah PP. Dermatitis herpetiformis: an evaluation of diagnostic criteria. Br J Dermatol 1974; 90: 137-146.

166. Chorzelski TP. Beutner EH, Jablonska S, Blaszczyk M, Triftshauser C. Immunofluorescence studies in the diagnosis of dermatitis herpetiformis and its differentiation from bullous pemphigoid. J Invest Dermatol 1971; 56: 373-80.

167. Fry L, Walkden V, Wojnarowska F et al. Comparison of IgA positive and IgA negative dapsone responsive dermatoses. Br J Dermatol 1980; 102: 371-82.

168. Zone JJ, Carioto LA, La Salle BA et al. Granular IgA is decreased or absent in never involved skin in dermatitis herpetiformis. Clin Res 1985; 33: 159A.

169. Zone JJ, Sayre LA, Meyer LJ. Granular IgA is quantitatively greater in perilesional skin than in adjacent uninvolved skin in patients with dermatitis herpetiformis. Clin Res 1987; 35: 253A.

170. Seah PP, Fry L, Hoffbrand AV et al. Tissue antibodies in dermatitis herpetiformis and adult coeliac disease. Lancet 1971; i: 834-36.

171. Unsworth DJ, Leonard JN, McMinn RMH et al. Antigliadin antibodies and small intestinal mucosal damage in dermatitis herpetiformis. Br J Dermatol 1981; 105: 653-58.

172. Chorzelski TP, Sulej J, Tchorzewska H et al. IgA class endomysium antibodies in dermatitis herpetiformis and coeliac disease. Ann NY Acad Sci 1983; 420: 325-34.

173. Beutner EH, Chorzelski TP, Jordon RE. Autosensitization in pemphigus and bullous pemphigoid. Springfield, IL: Charles C Thomas. 1970. Pp.160-72.

174. Jefferies R, Riemer CB, Skavariz F et al. Evaluation of monoclonal antibodies having specificity for human IgG subclasses: results of an IUIS/WHO collaborative study. Immunol Lett 1985; 10: 223-52.

175. Scaletta LJ, Occhino JC, MacCallum DK, Lillie JH. Isolation and immunologic identification of basement membrane zone antigens from human skin. Lab Invest 1978; 39: 1-9.

176. Lowrey OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951; 193: 265-75.

177. Laemmli UK. Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature 1970; 227: 680-85.

178. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979; 76: 4350-54.

179. Cormane RH, Petzoldt D. Immunofluorescence studies on the affinity of pemphigus antibodies to epithelial intercellular substance. Dermatologica 1970; 140: 1-8.

180. Sison-Fonacier I, Bystryn JC. Regional variations in antigenic properties of skin. J Exp Med 1986; 164: 2125-30.

181. Zhu X-J, Bystryn JC. Heterogeneity of pemphigoid antigens. J Invest Dermatol 1983; 80: 16-20.

182. Feibelman C, Stolzner G, Provost TT. Superior sensitivity of monkey oesophagus in the determination of pemphigus antibody. Arch Dermatol 1981; 117: 561-62.

183. Chorzelski TP, Beutner EH. Factors contributing to occasional failures in demonstration of pemphigus antibodies by the immunofluorescent test. J Invest Dermatol 1969; 53: 188-91.

184. Goldberg D, Sabolinski M. Bystryn JC. Bullous pemphigoid antibodies. Arch Dermatol 1985; 121: 1137-40.

185. Hamn G, Wozniak KD. Bullous pemphigoid antigen concentration in normal human skin in relation to body area and age. Arch Dermatol Res 1988; 280: 416-19.

186. Ioannides D, Hytiroglou P, Phelps RG, Bystryn JC. Regional variation in the expression of pemphigus foliaceus, pemphigus erythematosus and pemphigus vulgaris antigens in human skin. J Invest Dermatol 1991; 96: 159-61.

187. Wick G, Beutner EH. Quantitative studies of immunofluorescent staining. III. Comparison of different antigens in an indirect immunofluorescent staining for human IgG using the basement membrane zone antibodies of bullous pemphigoid. Immunology 1969; 16: 149-56.

188. Muller HK, Sutherland RC, Jamieson WJ et al. Autoantibodies in bullous pemphigoid. Med J Aust 1971; 1: 429-31.

189. Gligora M. Epithelium of human palatal tonsil for new test antigen in indirect immunofluorescence examination. Dermatologica 1977; 155: 108-14.

190. Kransy S, Beutner EH, Chorzelski TP. Specificity and sensitivity of indirect and direct immunofluorescent findings in the diagnosis of pem-

phigus. In: Beutner EH, Chorzelski TP, Kumar V (eds) Immunopathology of the Skin, 3rd ed. New York: Wiley and Sons. 1987. Pp.207-47.

191. Judd KP, Mescon H. Comparison of different epithelial substrates useful for indirect immunofluorescence testing of sera from patients with active pemphigus. J Invest Dermatol 1979; 72: 314-16.

192. Sabolinski ML, Beutner EH, Kransy S. Substrate specificity of anti-epithelial antibodies of pemphigus vulgaris and pemphigus foliaceus sera in immunofluorescent tests on monkey and guinea pig oesophagus sections. J Invest Dermatol 1987; 88: 545-49.

193. Sams WM Jr, Schur PH. Studies of the antibodies in pemphigoid and pemphigus. J Lab Clin Med 1973; 82: 249-54.

194. Brooks WS, Lee J, Abell G, Deng JS. Comparison of IgG subclasses and complement binding activity in patients with bullous pemphigoid and pemphigus (abstr). J Invest Dermatol 1987; 88: 479.

195. Yamada H, Hashimoto T, Nishikawa T. IgG subclasses of intercellular and basement membrane zone antibodies: The relationship to the capability of complement fixation. J Invest Dermatol 1989; 92: 585-87.

196. Flotte T, Baird L. Immunoglobulin light and heavy chain isotypes in skin diseases: restricted distribution in bullous pemphigoid and linear IgA bullous dermatoses. J Immunol 1986; 136: 491-96.

197. Hadi SM, Barnetson R, Gawkrodger DJ, Saxena U, Bird P, Merrett TG. Clinical, histological and immunological studies in 50 patients with bullous pemphigoid. Dermatologica 1988; 176: 6-17.

198. Borsos T, Rapp HJ. Complement fixation on cell surfaces by 19S and 7S antibodies. Science 1965; 150: 505-06.

199. Mooney E, Gammon Wr. Heavy and light chain isotypes of epidermolysis bullosa acquisita antibodies (abst). J Invest Dermatol 1987; 88: 508.

200. Adler TR, Beall GN, Cord JG, Heiner DC, Subharwal UK. Studies of complement activation and IgG subclass restriction of antithyroglobulin. Clin Exp Immunol 1984; 56: 383-89.

201. Robboy SJ, Lewis EJ, Schur PH, Colman RW. Circulating anti-coagulants to factor VIII: immunochemical studies and clinical response to factor VIII concentrates. Am J Med 1970; 49: 742-52.

202. Husby S, Oxelius V-A, Teisner B, Jensenius JC, Svehag SE. Humoral immunity to dietary antigens in healthy adults. Occurrence, isotype and IgG subclass distribution of serum antibodies to protein antigens. Int Arch Allergy Appl Immun 1985; 77: 416-22.

203. Aalberse RC, Vander Gaeg R, Vanleeuwen J. Serological aspects of IgG_4 antibodies. I. Prolonged immunization results in an IgG_4 restricted response. J Immunol 1985; 130: 722-26.

204. Perelmutter I. IgG_4 and the immune system. Clin Rev Allergy 1983; 1: 267-88.

205. Roberts JL, Wyatt RJ, Schwatz MM, Lewis EJL. Differential characteristics of immune-bound antibodies in diffuse proliferative and membranous forms of lupus glomerulonephritis. Clin Immunol Immunopathol 1983; 29: 223-41.

206. Schmidt I, Ullrich B, Rule A, Schaumburg-Lever G, Leblanic C. Ultrastructural localization of in vivo-bound complement in bullous pemphigoid. J Invest Dermatol 1975; 65: 217-19.

207. Jordon RE. Complement activation in pemphigus and bullous pemphigoid. J Invest Dermatol 1976; 67: 366-71.

208. Dahl MV, Falk RJ, Carpenter R, Michael AF. Deposition of the membrane attack complex of complement in bullous pemphigoid. J Invest Dermatol 1984; 82: 132-35.

209. Jordon RE, Schroeter AL, Good RA, Day NK. The complement system in bullous pemphigoid. II. Immunofluorescent evidence for both classical and alternate pathway activation. Clin Immunol Immunopathol 1975; 2: 307-14.

210. Jordon RE, Day NK, Sams WM Jr, Good RA. The complement system in bullous pemphigoid. I. Complement and component levels in sera and blister fluids. J Clin Invest 1973; 52: 1207-14.

211. Gammon WR, Merritt CC, Lewis DM. An in vitro model of immune complex-mediated basement membrane zone separation caused by pemphigoid antibodies, leucocyte and complement. J Invest Dermatol 1982; 78: 285-90.

212. Naito K, Morioka S, Ogawa H. The pathogenic mechanisms of blister formation in bullous pemphigoid. J Invest Dermatol 1982; 79: 303-06.

213. Naito K, Morioka S, Ikeda S, Ogawa H. Experimental bullous pemphigoid in guinea pigs: the role of pemphigoid antibodies, complement and migrating cells. J Invest Dermatol 1984; 82: 227-30.

214. Sams WM, Gammon WR. Mechanism of lesion production in pemphigus and pemphigoid. J Am Acad Dermatol 1982; 6: 431-49.

215. Wintraub BU, Mihm MC, Goetzl EJ, Soter NA, Austen KF. Morphologic and functional evidence for release of mast cell products in bullous pemphigoid. N Engl J Med 1978; 298: 417-21.

216. Gammon WR, Inman AO III, Wheeler CE Jr. Differences in complement-dependent chemotactic activity generated by bullous pemphigoid and epidermolysis bullosa acquisita immune complexes: demonstration by leucocyte attachment and organ culture methods. J Invest Dermatol 1984; 83: 57-61.

217. David M, Katzenelson V, Hazaz B, Ben-Chetrit A, Sandbank M. Determination of IgG subclasses in patients with pemphigus with active disease and in remission. Arch Dermatol 1989; 125: 787-90.

218. Rucco J, Rossi A, Argenziano G et al. Pathogenecity of the intercellular antibodies and their periodic removal from the circulation by plasmapheresis. Br J Dermatol 1978; 98: 237-41.

219. Moncada B, Kettelsen S, Hernandez-Moctezuma JL, Ramirez F. Neonatal pemphigus vulgaris: role of passively transferred pemphigus antibodies. Br J Dermatol 1982; 106: 465-68.

220. Michel B, Ko CS. Effect of pemphigus or bullous pemphigoid sera and leucocytes on normal human skin in organ culture. An in vitro model for the study of bullous diseases. J Invest Dermatol 1974; 62: 541a-42a.

221. Schiltz JR, Michel B. Production of epidermal acantholysis in normal human skin in vitro by the IgG fraction from pemphigus serum. J Invest Dermatol 1976; 67: 254-60.

222. Farb RM, Dykes R, Lazarus GS. Antiepidermal cell surface pemphigus antibody detaches viable epidermal cells from culture plates by activation of proteinase. Proc Natl Acad Sci USA 1978; 75: 459-63.

223. Hashimoto K, Shafran KM, Webber PS, Lazarus GS, Singer KH. Anti-cell surface pemphigus autoantibody stimulates plasminogen activator activity of human epidermal cells. J Exp Med 1983; 157: 259-72.

224. Anhalt GJ, Labib RS, Voorhees JJ et al. Induction of pemphigus in neonatal mice by passive transfer of IgG from patients with the disease. N Engl J Med 1982; 306: 1189-96.

225. Anhalt GJ, Patel HP, Labib RS, Diaz LA, Proud D. Dexamethasone inhibits plasminogen activator activity in experimental pemphigus in vivo but does not block acantholysis. J Immunol 1986; 136: 113-17.

226. Jordon RE, Schroeter AL, Rogers RS, Perry HO. Classical and alternate pathway activation of complement in pemphigus vulgaris lesions. J Invest Dermatol 1974; 63: 256-59.

227. Van Joost T, Cormane RH, Pondman KW. Direct immunofluorescent study of the skin on occurrence of complement in pemphigus. Br J Dermatol 1972; 87: 466-74.

228. Jordon RE. Complement activation in pemphigus. J Invest Dermatol 1980; 74: 357-59.

229. Jordon RE, Day NK, Luckasen JR, Good RA. Complement activation in pemphigus vulgaris blister fluid. Clin Exp Immunol 1973; 15: 53-63.

230. Jordon RE, Sams WM, Diaz G, Beutner EH. Negative complement immunofluorescence in pemphigus. J Invest Dermatol 1971; 57: 407-10.

231. Nishikawa T, Kurihara S, Harrada J, Sugawara M, Hatano H. Capability of fixation of pemphigus antibodies in vitro. Arch Dermatol Res 1977; 260: 1-6.

232. Cerio R, Bhogal BS, Kelly S, Black MM. Differences in IgG subclasses in herpes gestationis, pemphigus and bullous pemphigoid. J Cut Path 1987; 14: 352.

233. Shehade A, Layton GT, Stanworth DR. IgG_4 and IgE antibodies in atopic dermatitis and urticaria. Clin Exp Dermatol 1988; 13: 393-96.

234. Nakegawa T, Migamoto T. The role of IgG_4 as blocking antibodies in asthmatics and beekeepers. Int Archs Allergy Appl Immun 1985; 77: 204-05.

235. Gammon WR, Briggaman RA, Woodley DT et al. Epidermolysis bullosa acquisita - a pemphigoid-like disease. J Am Acad Dermatol 1984; 11: 820-32.

236. Kelly SE, Wojnarowska F. The use of chemically split tissue in the detection of circulating antibasement membrane zone antibodies in bullous pemphigoid and cicatricial pemphigoid. Br J Dermatol 1988; 118: 31-40.

237. Woodley DT, Sauder D, Talley MJ et al. Localization of basement membrane components after dermal-epidermal junction separation. J Invest Dermatol 1983; 81: 149-53.

238. Gammon Wr, Briggaman RA, Inman AO et al. Differentiating anti-lamina lucida and anti-sublamina densa anti-basement membrane zone antibodies by indirect immunofluorescence on 1M NaCl separated skin. J Invest Dermatol 1984; 82: 139-44.

239. Logan RA, Bhogal B, Das SK, McKee PM, Black MM. Localization of bullous pemphigoid antibody in indirect immunofluorescence study of 228 cases using a split skin technique. Br J Dermatol 1987; 117: 471-78.

240. Zhu Y-J, Niimi Y, Bystryn JC. Epidermolysis bullosa acquisita incidence in patients with basement membrane zone antibodies. Arch Dermatol 1990; 126: 171-74.

241. Fine JD, Tyring S, Gammon WR. The presence of intra-lamina lucida blister formation in epidermolysis bullosa acquisita: possible role of leukocytes. J Invest Dermatol 1989; 92: 17-32.

242. McCuaig CC, Chan LS, Woodley DT, Rasmussen JE, Cooper KD. Epidermolysis bullosa acquisita in childhood: differentiation from hereditary epidermolysis bullosa. Arch Dermatol 1989; 125: 944-49.

243. Barthelemy H, Kanitakis J, Cambazard F et al. Epidermolysis bullosa acquisita - mapping of antigenic determinants by an immunofluorescent technique. Clin Exp Dermatol 1986; 11: 378-86.

244. Gammon WR, Kowalewski C, Chorzelski TP, Kumar V, Briggaman RA, Beutner EH. Direct immunofluorescence studies of sodium chloride-separated skin in the differential diagnosis of bullous pemphigoid and epidermolysis bullosa acquisita. J Am Acad Dermatol 1990; 22: 664-70.

245. Wuepper KD. Repeat direct immunofluorescence to discriminate pemphigoid from epidermolysis bullosa acquisita. Arch Dermatol 1990; 126: 1365.

246. Labib RS, Anhalt GJ, Patel HP, Mutasim DF, Diaz LA. Molecular heterogeneity of the bullous pemphigoid antigens as detected by immunoblotting. J Immunol 1986; 136: 1231-35.

247. Zhu X-J, Niimi Y, Bystryn JC. Molecular identification of major and minor bullous pemphigoid antigens. J Am Acad Dermatol 1990; 23: 876-80.

248. Bernard P, Didierjean L, Denis F, Stuart J-H, Bonnetblanc J-M. Heterogeneous bullous pemphigoid antibodies: detection and characterization by immunoblotting when absent by indirect immunofluorescence. J Invest Dermatol 1989; 92: 171-74.

249. Mueller S, Klaus-Kovtun V, Stanley JR. A 230 Kd basic protein is the major bullous pemphigoid antigen. J Invest Dermatol 1989; 92: 33-38.

250. Zhu X-J, Niimi Y, Bystryn JC. Identification of a 160 Kd molecule as a component of the basement membrane zone and as a minor bullous pemphigoid antigen. J Invest Dermatol 1990; 94: 817-21.

251. Meyer LJ, Taylor TB, Kadunce DP, Zone JJ. Two groups of bullous pemphigoid antigens are identified by affinity purified antibodies. J Invest Dermatol 1990; 94: 611-16.

252. Robledo MA, Kim S-C, Korman NJ, Stanley JR, Labib RS, Futamura S, Anhalt GJ. Studies of the relationship of the 230 Kd and 180 Kd bullous pemphigoid antigens. J Invest Dermatol 1990; 94: 793-97.

253. Stanley JR, Rubenstein N, Klaus-Kovtun V. Epidermolysis bullosa acquisita antigen is synthesized by both human keratinocytes and human dermal fibroblasts. J Invest Dermatol 1985; 85: 542-45.

254. Niimi Y, Zhu X-J, Bystryn JC. Identification of basement membrane zone antigens defined by antibodies that react to both the epidermal and dermal side of 1M sodium chloride split skin. J Invest Dermatol 1991; 97: 312-17.

255. Chorzelski TP, Beutner EH, Sulej J et al. IgA anti-endomysium antibody: a new immunological marker of dermatitis herpetiformis and coeliac disease. Br J Dermatol 1984; 111: 395-402.

256. Kumar V, Beutner EG, Chorzelski TP. Distribution of monkey oesophagus antigens reactive with IgA class antibodies in the sera of dermatitis herpetiformis patients. Arch Dermatol Res 1984; 276: 293-96.

257. Beutner EG, Chorzelski TP, Kumar V et al. Sensitivity and specificity of IgA class anti-endomysial antibodies for dermatitis herpetiformis and findings relevant to their pathogenic significance. J Am Acad Dermatol 1986; 15: 464-73.

258. Weinstein WM. Latent coeliac sprue. Gastroenterology 1974; 66: 489-93.

259. Chorzelski TP, Jablonska S, Beutner EG, Kumar V, Sulej J, Leonard JN. Anti-endomysial antibodies in dermatitis herpetiformis and coeliac disease. In: Beutner EH, Chorzelski TP, Kumar V (eds) Immunopathology of the Skin, 3rd ed.. New York: Wiley and Sons. 1987. Pp.477-82.

260. Meurer M, Reff P, Bułgin-Wolff A et al. Circulating IgA class endomysium antibodies in dermatitis herpetiformis: correlation with enteropathy and gluten-free diet (abst). J Invest Dermatol 1985; 84: 453.

261. Accetta P, Kumar V, Beutner EH et al. Anti-endomysial antibodies. Arch Dermatol 1986; 122: 459-62.

262. Chorzelski TP, Jablonska S, Chadzynska M et al. IgA endomysium antibody in children with dermatitis herpetiformis treated with gluten-free diet. Pediatr Dermatol 1986; 3: 291-94.

263. Reunala T, Chorzelski TP, Viander M et al. IgA anti-endomysial antibodies in dermatitis herpetiformis: correlation with jejeunal morphology, gluten-free diet and anti-gliadin antibodies. Br J Dermatol 1987; 117: 185-91.

264. Peters MS, McEvoy MT. IgA anti-endomysial antibodies in dermatitis herpetiformis. J Am Acad Dermatol 1989; 21: 1225-31.

265. Leonard JN, Chorzelski TP, Beutner EH et al. IgA anti-endomysial antibody detection in the serum of patients with dermatitis herpetiformis following gluten challenge. Arch Dermatol Res 1985; 277: 349-51.

266. Chorzelski TP, Rosinka D, Beutner EH et al. Aggressive gluten challenge of dermatitis herpetiformis cases converts them from seronegative to seropositive for IgA class endomysial antibodies. J Am Acad Dermatol 1988; 18: 672-78.

267. Kumar V, Jain N, Beutner EH, Chorzelski TP. Detection of anti-gliadin antibodies in bullous diseases and their recognition of similar antigenic polypeptides. Int Arch Allergy Appl Immunol 1987; 83: 155-59.

268. Kieffer M, Barnetson RStC. Increased gliadin antibodies in dermatitis herpetiformis and pemphigoid. Br J Dermatol 1983; 108: 673-78.

269. Kumar V, Beutner EH, Lerner A et al. Comparison of disease specificity of endomysial and anti-gliadin antibodies. In: Beutner EH, Chorzelski TP, Kumar V (eds) Immunopathology of the Skin, 3rd ed. New York: Wiley and Sons. 1987. Pp.483-88.

270. Ljunghall K, Scheynius A, Forsum U. Circulating reticulin antibodies of IgA class in dermatitis herpetiformis. Br J Dermatol 1979; 100: 173-76.

271. Hölström O. Comparison of IgA class reticulin and endomysium antibodies in coeliac disease and dermatitis herpetiformis. Gut 1989; 30: 1225-30.

272. Kumar V, Hemedinger E, Chorzelski TP et al. Reticulin and endomysial antibodies in bullous diseases: comparison of specificity and sensitivity. Arch Dermatol 1987; 123: 1179-82.

273. Kumar V, Beutner EH, Chorzelski TP. Anti-endomysial antibody: useful serological indicator of dermatitis herpetiformis. Arch Dermatol Res 1987; 279: 454-58.

GLASGOW UNIVERSITY LIBRARY