Immunofluorescence techniques in dermatology involve the detection of autoantibodies directed against target antigens in human skin. Direct and indirect immunofluorescence, salt split skin technique, immunoelectron microscopy and immunoblotting remain relevant and often first-line diagnostic tools. Recently developed enzyme linked immunosorbent assays (ELISA) which utilize recombinant proteins to detect serum antibodies demonstrate high sensitivity and specificity in many skin conditions. The ELISA using the NC16a domain of bullous pemphigoid antigen-2 (180 kDa) is an important adjunct tool in the diagnosis of bullous pemphigoid, mucous membrane pemphigoid, anti-epiligrin cicatricial pemphigoid, lichen planus pemphigoides and pemphigoid gestationis. The ELISA utilizing the NC1 domain of type VII collagen is relevant in the diagnosis of bullous systemic lupus erythematosus and epidermolysis bullosa acquisita. In dermatitis herpetiformis, the use of both the indirect immunofluorescence method to detect IgA anti-endomysial antibodies and the tissue transglutaminase, ELISA increases diagnostic sensitivity. In the diagnosis of necrotizing vasculitis, ELISAs using cardiolipin have been developed to detect antibodies in Henoch-Schonlein purpura, while ELISAs using proteinase-3 and myeloperoxidase were studied for the diagnosis of Wegener’s granulomatosis. The differential diagnosis of other immunofluorescence patterns like cytoid bodies, epidermal nuclear fluorescence are also discussed. (Dermatol Sinica 26: 191-227, 2008)

Key words: Pemphigoid, Epidermolysis bullosa acquisita, Lichen planus, Lupus erythematosus, Linear IgA bullous dermatosis, Dermatitis herpetiformis

I. BASEMENT MEMBRANE ZONE DEPOSITS

A. BASEMENT MEMBRANE ZONE STAINING: LINEAR DEPOSITION

Bright continuous linear deposition of immunoreactants at the basement membrane zone (BMZ), particularly with a predominance of C3 staining, is characteristic of the pemphigoid group, which encompasses bullous pemphigoid (BP), cicatricial/mucous membrane pemphigoid (MMP) and pemphigoid gestationis (PG). The presence of a continuous broad linear band of predominantly IgG at the BMZ is characteristic of epidermolysis bullosa acquisita (EBA), however, the final distinction will be made through
salt-split skin, immunoblotting (IB) and enzyme linked immunosorbent assays (ELISA) techniques.

1. Pemphigoid group
   a. Bullous pemphigoid

BP is an acquired autoimmune blistering disease characterized clinically by tense blisters arising on either normal, erythematous or urticarial skin and heal without scarring. It usually occurs in elderly patients from 50-80 years of age, with a female predilection. Mucosal involvement is rarely present, and if seen, usually only affects the oral mucosa with a desquamative gingivitis. Histologically, there is a subepidermal blister with an eosinophil-predominant inflammatory infiltrate. Immunopathologically, BP is characterized by circulating IgG autoantibodies binding to the 230kDa (BPAg1, in 90% of patients) and 180 kDa (BPAg2 type XVII collagen) hemodesmosomal proteins. The 230kDa bullous pemphigoid antigen (BPAg1, BP230) is recognized by most BP sera using IB assay. IgG4 is the dominant immunoglobulin subtype.

Of 26 BP patient sera analyzed for circulating IgG and IgA anti-BMZ autoantibodies by indirect immunofluorescence (IIF), 100% had circulating IgG autoantibodies but an additional 35% also had circulating IgA autoantibodies binding to the epidermal side of salt split skin. This was confirmed with IB, where 7/9 BP sera had IgA autoantibodies binding to 180kDa, 230 kDa or both antigens, and even a 270-280kDa antigen. This however did not impact the clinical presentation. In some patients with BP, IgG antibodies to GST-4575 and other epitopes within the C-terminal portion of BP180 ectodomain were found as well.

Direct immunofluorescence (DIF) will usually show linear BMZ staining with C3 staining more intensely than IgG and sometimes IgM or IgE or fibrin will be found depositing along the BMZ. (Fig. 1) Vodegel et al describe this true linear staining as “n-serrated” on DIF, but would be found as well in MMP, anti-epiligrin cicatricial pemphigoid, p200 pemphigoid and linear IgA bullous dermatoses (LAD), corresponding with deposits in hemodesmosomes, lamina lucida or lamina densa. IIF will show anti-BMZ IgG autoantibodies in more than 70% of patients. Salt split skin will show staining on the epidermal (roof) side of the blister. Antigen mapping will reveal anti-BP antibodies binding to the roof of the blister, and anti-laminin and anti-type IV collagen antibodies staining the floor of the blister, confirming a split through the lamina lucida. IB of BP sera using 3 recombinant proteins representing the N-terminal domain, central rod domain and C-terminal domain of BP230 showed

Fig. 1
Bright thin continuous linear staining of C3 at the basement membrane zone reactions in a case of bullous pemphigoid. (fluorescence microscopy, original magnification x400)
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that sera from patients with BP sera reacted specifically to these recombinant proteins, but not with sera from patients with para-neoplastic pemphigus, pemphigus vulgaris or pemphigus foliaceus.\textsuperscript{13} IB or immunoprecipitation likewise demonstrate antibodies against a 180kDa BPAg2 protein.\textsuperscript{3, 4}

A study done comparing the sensitivity of IIF to IB assay for detection of circulating antibodies to BPAg1 and BPAg2 showed that of 263 BP patients, IIF detected 75\% of sera containing anti-BMZ antibodies, compared to 69\% of 182 cases by IB with BP antigens (51\% with BPAg1 and 26\% with combination BPAg1 and 2, and 23\% with BPAg2 alone). They found a good correlation between IIF and IB assay detection. Of 65 IIF negative sera, IB assay yielded a positive result in 46\%.\textsuperscript{4} IIF using guinea pig esophagus as substrate showed 19\% of normal elderly patients with autoantibodies against BMZ antigens in titers ranging from 40-320, compared to none in young individuals.\textsuperscript{14}

Barnadas \textit{et al} did a study using 1M salt split skin technique on 56 samples initially diagnosed on DIF as BP. Seventy-two (72\%) of biopsies showed staining on either the epidermal or both sides after splitting while 10\% of cases showed only floor deposition. Repeating DIF after conducting salt split in patients initially diagnosed as BP suggests that doing only DIF without a salt split may miss the diagnosis in 12\% of patients.\textsuperscript{15}

IMMUNOELECTRON MICROSCOPY (IEM) of BP

A study done by Karpouzis showed that direct or indirect immunoelectron microscopy (IEM) using diaminobenzidine determined the location of immune deposits in a majority of subepidermal autoimmune blistering diseases such as cicatricial pemphigoid, LAD or EBA.\textsuperscript{16} IEM findings in BP show cleavage within the lamina lucida and antibody deposits in the upper lamina lucida, close to the plasma membrane of basal keratinocytes immediately beneath the hemidesmosomal plaque.\textsuperscript{5, 17, 18, 19} In patients with anti-BPaAg 1(230 kDa) sera, antibodies are deposited only at the intracellular portion of hemidesmosomes at basal keratinocytes, strictly within the lamina lucida.\textsuperscript{19} Patients who have antibodies against the NC1a domain of BPAg2 (180kDa) show deposits extending from outer hemidesmosomal plaque to the upper lamina lucida.\textsuperscript{5}

DOUBLE ANTIBODY SANDWICH METHOD AND IGG ELISA

It has been demonstrated that the membrane proximal non-collagenous domain NC16a of BP180 is the immunodominant region targeted by BP autoantibodies. Commercially available ELISA systems for BP to detect autoantibodies directed against the NC16a ectodomain of BP180 (usually monomers) was compared to IF, which showed high sensitivity of 89\% and specificity of 98\% in 102 BP sera.\textsuperscript{20} The authors concluded that the BP180-NC16a ELISA is highly sensitive and specific, easy to perform, objective, semiquantitative and reliable. ELISA levels of anti-BP180 autoantibodies were found to be related to disease severity in BP.\textsuperscript{21} However, in BP, no correlation has been observed between IIF titers and ELISA values.\textsuperscript{20} Sitaru \textit{et al} used NC16a teramic antigen fragments of BP180 and found a sensitivity of 90\% and specificity of 98\% for BP and PG sera, with levels of autoantibodies against BP180 actually paralleling disease activity.\textsuperscript{22} Mariotti \textit{et al} developed an ELISA system using additional epitopes of the BP180 ectodomain (AA 1080-1107, AA 1331-1404) and combined then into a novel ELISA to GST-1080/GST-1331, resulting in 41\% of 78 BP sera testing positively. Using this in combination with traditional GST-NC16a, sensitivity increased from 82\% to 92\% in BP patients.\textsuperscript{23}
In 12 BP patients who on DIF showed positive C3 staining along the BMZ but negative IgG staining, serum was tested using either: a) double antibody sandwich method using IgG, IgG subclasses and light chains, in which all 12 sera showed BMZ staining using the IgG4 subclass; b) or a commercial ELISA using IgG1 and IgG3 subclasses, showing a high reactivity to both subclasses. They concluded that IgG negativity on DIF in some BP patients may be due to subthreshold IgG in skin specimens, limited reactivity of commercial DIF IgG conjugates to IgG subclass, and decreased sensitivity of traditional DIF compared to the double antibody sandwich method to detect IgG.

Asashima et al in 2006 developed an ELISA for soluble BAFF (B-cell activating factor belonging to the tumor necrosis factor (TNF) superfamily that regulates B-lymphocyte proliferation and survival. Increased levels of soluble BAFF have been found in autoimmune diseases like lupus erythematosus. Comparing with ELISA to BP180, patients with BP were found to have significantly elevated levels of serum BAFF, which increased before anti-BP180 antibody levels at the onset of BP, and likewise decreased with response to therapy. They concluded that BAFF may be a useful marker for early autoimmune activation and may trigger self-antigen driven autoreactive B cells in BP.

**IF OF EUKARYOTIC CELLS TO DETECT BP180**

Schmidt et al developed a very sensitive IF using Sf21 insect cells transfected with full-length BP180, which contrasts with the currently available prokaryotic recombinant BP180 fragments that lack conformation-dependent epitopes. They found this eukaryotic IF assay to be more sensitive that IIF on human skin substrate and IB using keratinocyte-derived BP180 ectodomain, recombinant BP180 NC16a, with a sensitivity of 89% in BP, 81% in PG and 84% in MMP, and high specificity, with all control sera negative.

**b. Cicatricial/mucous membrane pemphigoid**

Mucous membrane (cicatricial) pemphigoid is a clinically and immunopathologically heterogeneous group of acquired autoimmune subepidermal blistering diseases characterized by chronic recurrent tense blistering of skin as well as oral and ocular mucous membranes, resulting in significant scarring. Oral involvement may be seen in 85% of patients, ocular involvement in 65% and skin involvement in 25% of patients. MMP is a chronic progressive disease with significant morbidity due to scarring and functional loss, and prognosis may be associated with the site of involvement. Site, severity and disease progression determine treatment options, and high risk patients require systemic steroids and immunosuppressives (like cyclophosphamide and azathioprine, IVIG) and multi-specialty care. The disease is chronic and recurrent and lasts from months to up to 25 years. Histopathology shows a subepidermal bulla with a mixed inflammatory infiltrate, and late recurrent lesions with scarring will reveal subepidermal fibrosis, and sclerosis of adnexal structures.

Sera from MMP patients may target different autoantigens, and 10 different epithelial BMZ antigens have been recognized including: BP230 (BPAg1), BP180 (BPAg2, type XVII collagen, recognized by some studies as the major autoantigen in MMP especially C-terminal stretches of BP180 ectodomain targeted by IgG and IgA antibodies) and its soluble ectodomains 120kDa and 97kDa; laminin-332/laminin-5 (3βα3γ2 chains), laminin 6 (β3 chain), type VII collagen, β4-integrin subunit, and other unknown antigens (uncein, 45-, 168 and 120-kDa)
epithelial proteins). Cicatricial pemphigoid patients studied using DIF, IIF, IB using human skin and esophagus substrates revealed significant reactivity of circulating anti-BMZ IgG and IgA autoantibodies to BP180 (BP180) and 97kDa linear IgA disease (LAD) antigen. It has been suggested that MMP patients with both IgG and IgA autoantibodies to BMZ antigens have more severe and persistent disease. Forty percent (40%) of MMP sera was shown to contain IgA antibodies to GST BP915.

Passive transfer methods into neonatal mice have shown antibodies to BP180 and laminin 5 to induce blister formation, indicating that these are pathogenic autoantibodies in vivo. Factors possibly involved in the pathogenesis of MMP include: increased occurrence of HLA-DQB*0301, DRB*04, DRB1*11 alleles, complement activation, cellular immunity and cytokines, and epitope spreading.

DIF in MMP will show continuous linear deposition of any 1 or combination of anti-BMZ antibodies: C3 (in 48%) and IgG (in 90%) of patients at the BMZ. Rarely, IgA and fibrin will also stain the BMZ. DIF of MMP does not distinguish it from BP, LAD or EBA, and clinical distinction and other immunopathologic tests should be utilized. IIF is usually negative but may reveal low titers of IgG BMZ antibodies in 15-30% of patients. None show circulating IgA anti-BMZ antibodies. Indirect salt split skin is a sensitive test and may present with 3 patterns reflecting heterogeneity of autoantibodies: deposits on the epidermal side of the blister, combined in-anti-epiligrin CP, deposits binding to the dermal side of the split. Antigen mapping will reveal cleavage at the level of deep lamina lucida at the junction with the lamina densa, resulting in both anti-BP and anti-laminin antibodies binding to the epidermal side of the blister split. IB will reveal staining to BP180 (230 kDa) and in a subset of patients to the NC16a domain of BP180 (180 kDa). On IEM, autoantibody deposits localize to the deep lamina lucida at the interface between the lamina lucida to and below the lamina densa. Anchoring fibrils and other connective tissue elements in the dermis are well preserved, but some authors reported disappearance, disruption or duplication of basal lamina, possibly accounting for the clinical scarring.

IB using epidermal extracts showed 3/14 MMP patients had IgG and C3 autoantibodies to BP230, and 1/14 to BP180. None had autoantibodies to type VII collagen in dermal extracts. Oyama et al found a majority of MMP patients had IgG (75%) and/or IgA (51%) antibodies to BP180 and its soluble ectodomains (120 kDa and 97 kDa, usually related to more severe clinical phenotype), while IgG targeted BP230 in 27%, β4 integrin (related to ocular involvement) in 21% and laminin-5 in 2%, and relation of IgG/IgA reactivity to HLA class II alleles DQB1*0301, DRB*04, and DRB1*11.

Immunoprecipitation studies using biosynthetically radiolabeled extracts from cultured human keratinocytes showed that all patients showing a dermal pattern of staining on salt split skin immunoprecipitated laminin-5 (α3β3γ2) and 6 (α3β1γ1). Patients with sera staining combined or epidermal sides immunoprecipitated non-specific 150 kDa and 110kDa polypeptides. Dermal extracts for BP180 NC16a and fusion proteins representing C-terminal portion of BP180 ectodomains were expressed as glutathione S transferase fusion proteins and affinity purified using glutathione agarose, and used for IB and ELISA for BP180. Two of 14 MMP sera reacted with BP180 NC16a ELISA.

**ANTI-EPILIGRIN CICATRICIAL PEMPHIGOID (AECP)**

Anti-epiligrin cicatricial pemphigoid (AECP) is a subset of mucous membrane
pemphigoid clinically indistinguishable from MMP, but is characterized by IgG anti-BMZ autoantibodies directed against laminin-332 (L-332) (more commonly known as laminin-5, and other terms including epiligrin, ka-linin, nicien, BM600), and associated with an increased relative risk for solid cancers such as ovarian and colonic cancers and lymphomas (with a relative risk of approximately 7). Clinical features of AECP include: chronic subepidermal mucosal blistering mostly on oral mucosa; continuous deposition of IgG and/or C3 on BMZ on DIF; and circulating IgG autoantibodies that bind to the dermal side of a salt split skin and immunoprecipitate with L-332 from extracts of biosynthetically labeled human keratinocytes.

Laminin-332 or laminin-5 is a set of disulfide-linked polypeptides which is an anchoring filament-lamina densa associated heterotrimer, consisting of α3β3γ2. It was found that most AECP patients autoantibodies bind to the α3 subunit of this protein, and some have antibodies to laminin β3 or both β3 and γ2. Autoantibodies have been proven to be pathogenic through passive transfer into neonatal mice, resulting in subepidermal bullous disease identical to AECP.

DIF of perilesional skin or mucous membrane demonstrates linear deposits of IgG and C3 at the BMZ. IIF studies show binding of IgG autoantibodies to the dermal side of salt split human skin. IB was done using laminin-332 derived from the extracellular matrix (ECM) of cultured human keratinocytes and reacted with sera from AECP patients, and showed 30/32 AECP patient sera tested reacting to one of more subunits of L-332. Cultured human keratinocytes produce significant amounts of L-332 and incorporate all subunits of this heterotrimer into ECM around proliferating cells. This substrate is regarded as the most sensitive for detecting autoantibodies in AECP patients. IB using this substrate showed that sera from AECP patients bound to 200kDa and 165kDa subunits of laminin α3. Immunoprecipitation studies utilize subconfluent monolayers of human keratinocytes biosynthetically radiolabeled with S-methionine, which are immunoprecipitated with patient sera and studied using polyacrylamide gel electrophoresis and fluorography. IP is the most sensitive technique known for detection of autoantibodies in AECP patients which recognize laminin-332.

L-332 in the ECM of human keratinocytes was isolated and used for antigen capture of specific IgG by ELISA. An early ELISA for L-332 was developed by Bekou et al in 2005, which yielded a sensitivity of 40.3% and specificity of 88.2% but detected L-332 autoantibodies in 40% of BP patients as well. The lack of specificity attributed to the antigen source which was a conditioned medium of squamous cell carcinoma-25 cells. A more recent ELISA was refined by Lazarova et al using L-332 derived from purified extracellular matrix of cultured human keratinocytes in neonatal foreskins, and showed great diagnostic accuracy, achieving a high sensitivity (91%) and specificity (98%) for detecting IgG (predominant subclass IgG4) autoantibodies reactive against L-332. They found that almost all patients with AECP had significant levels of IgG4 reactive with L-332, and lower levels of IgG 1, 2 or 3. In this study only 10% of BP sera tested reacted to L-332 by IgG4 ELISA, and none with pemphigus vulgaris, pemphigus foliaceus, or healthy patients. They concluded that this ELISA was practical and useful as a screening tool, and useful to accurately identify patients who were suspected of having AECP warranting testing with IB or immunoprecipitation to detect anti-L-332 IgG autoantibodies, further emphasizing the need for using a combination of diagnostic immunopathology tests for difficult blistering cases.
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c. PEMPHIGOID GESTATIONIS (PG)

Pemphigoid (herpes) gestationis, often described as a subset of BP, is a rare autoimmune subepidermal blistering disorder associated with pregnancy that usually occurs in the second or third trimester of pregnancy or immediately postpartum. It presents clinically as pruritic erythematous urticarial plaques evolving into tense blisters, usually affecting the periumbilical area, then legs, trunk, thighs and back, with mucosal involvement in 15% that heals without scarring. It carries some fetal risks such as prematurity, miscarriage and low birth weight. Transient blistering in neonates due to passive transfer of circulating autoantibodies have occurred in 5-10% of PG patients. Histopathology will reveal a subepidermal blister with eosinophils and eosinophilic spongiosis. Disease usually resolves gradually after delivery, but duration may range from months to years and usually recurs with subsequent pregnancies, with an earlier onset and more aggressive disease course on recurrence. PG is usually treated with systemic steroids, potent topical steroids, dapsone and antihistamines, and rarely immunosuppressants like azathioprine and tetracycline.

By epitope mapping, autoimmune response in PG is comprised of IgG autoantibodies targeting the extracellular NC16a domain of BP180, which has been identified as the primary target antigen BP and PG. IgG 1 and IgG 3 are predominant subclasses of anti-BP180 NC16a autoantibodies in PG. IgA reactivity to C-terminal portion of BP180 ectodomain has been found in 1 patient. Passive transfer studies of pathogenic antibodies to the NC16a portion of the ectodomain of BP180 induced blisters in neonatal mice and hamster BP models and other studies found that antibodies to the NC16a domain triggered expression and secretion of inflammatory mediators when incubated with cultured keratinocytes. PG has been associated with presence of predominant histocompatibility molecules human leukocyte antigens HLA-DR3 and HLA-DR4 and is genetically distinct from BP, which is associated with HLA-DQ3.

DIF in PG is the most sensitive and specific test which reveals strong linear deposition of C3 (IgG in 30%, and rarely IgA, IgM, C1 and C4) along the BMZ in most or all PG patients. IIF using monkey esophagus or human salt split skin show linear deposition of anti-C3 BMZ antibodies, but is less sensitive than DIF. IIF showed linear binding of antibodies to the roof/epidermal side of a salt split skin specimen in 87% using C3 and 82% using IgG in 82 PG patients. Indirect complement-fixing IF techniques show that most patients’ serum has complement-fixing IgG directed against BMZ antigen. IB studies have shown that 90% of PG sera have antibodies that target BP180. Only a few patients will be positive for HG factor (IgG1 subclass is predominant), however this test is not very sensitive. IB has also shown that 93% of PG sera are positive for autoantibodies to the NC16a domain. IB using epidermal extracts containing soluble 120 kDa ectodomain of BP180 (LAD-1 antigen), as well as recombinant fusion proteins (GST-NC16a, GST-4575, GST BP915, GSP BP963 and BST BP-1050 expressed in E.coli) showed presence of IgA autoantibodies to recombinant C-terminal BP180 ectodomain fragments GST 4574 and GST-BP915 (no IgG reactivity was noted). In PG, the immunoblot assay is more sensitive in detecting anti-BP180 antibodies with recombinant BP180 than BP180 extracted from epidermis or keratinocytes cultures.

A commercially available ELISA to NC16a of BP180 (as the major epitope of PG) was tested in 82 PG patients. Recombinant proteins encompassing the NC16a portion of the extracellular domain of BP180
(Medical and Biological Laboratories Co Ltd, Nagoya, Japan) was used. Using a cutoff value of 10, Powell et al determined ELISA to NC16a had a sensitivity was 96% and specificity was 96% in PG patients. Diagnostic accuracy was determined to be 99%. Previous ELISA studies using these recombinant portions of BP180 extracellular domain showed that 92% of 37 PG and 79% of 43 BP sera reacted to 1 or all 4 epitopes clustered in a 22 amino acid region of the NC16a domain of BP180. Other studies to this NC16a ELISA showed sensitivity ranging from 53% to 92% and specificity from 97-100% when used in PG patients. These studies found that ELISA values showed correlation of levels with clinical course of disease activity, and may be used to monitor autoantibody levels of anti-BP180 especially when tapering corticosteroids to suppress antibody production to prevent recurrence. Powell et al concluded that the NC16a ELISA is more sensitive than IF using either IgG or C3 technique alone, but is less sensitive than the traditional combination of both IIF techniques, which has a sensitivity of 99%. IIF and ELISA titers were found to have no correlation. False negative ELISA results and lack of correlation between IIF and ELISA values have been speculated to be due to conformational differences between the 2 assays or reactivity of antibodies to other BMZ antigenic epitopes, outside of the NC16a domain of BP180.

In PG, due to low incidence of circulating autoantibodies, DIF remains the gold standard and should be performed in pregnant patients with persistent urticarial or bullous lesions. Castro et al found only 1 of 7 PG patients positive for autoantibodies to BP180 using the ELISA test to BP180 but none to ELISA for desmogleins 1 and 3. For rapid routine diagnosis, the NC16a domain of BP180 ELISA is a highly sensitive and specific tool that allows testing of many PG sera, and results in objective and semiquantitative analysis of NC16a specific antibodies. Likewise, frequent testing with NC16a BP180 ELISA index facilitates treatment planning, if clinical activity is correlated to serum titers of anti BP180 autoantibodies.

d. DRUG INDUCED BULLOUS PEMPHIGOID

Common drugs that may trigger BP include penicillamine, furosemide, captopril, ampicillin, cephalexin, PUVA therapy, chloroquine and sulfasalazine. Drug induced BP may have identical clinical, histologic, DIF (linear IgG, IgM and C3 along BMZ, split skin showing epidermal staining, IgG antibodies directed against BP230) and IIF findings (split skin reveals IgG antibodies binding to epidermal side). Rarely, intercellular IgG deposits may be seen on DIF. IB has identified BPAG 1 and 2 as the target antigens as well.

2. Epidermolysis Bullosa Acquisita (EBA)

EBA is an autoimmune blistering disease characterized by autoantibodies against type VII collagen. Two variants of EBA are recognized: 1) classic EBA, presenting with
trauma induced blisters; and 2) inflammatory EBA, with acute blisters on an erythematous base. Clinically, it presents usually in older patients between the ages of 45 to 80. It is characterized by skin fragility, and recurrent tense blisters that may be spontaneous but are usually induced by mechanical trauma, and lesions which may heal with atrophic scars and milia formation. Oral and ocular mucosa may be affected in 50% of cases. Predisposition to EBA has a strong association with the HLA-DR2 allele. Diseases such as inflammatory bowel disease, diabetes and thyroiditis, and chronic hepatitic C infection have been associated with EBA, and is suggestive of altered immune status and debility. Histopathology shows subepidermal blisters which may be non-inflammatory or have a predominantly neutrophilic dermal infiltrate.

DIF will show linear deposits of IgG and C3 along the dermoeidermal junction. It has a chronic and relentless course, with scarring and milia formation and is relatively resistant to therapy. High dose steroids and immunosuppressives like azathioprine, methotrexate, cyclophosphamide, cyclosporine, photopheresis or IVIG may be tried. Relapse may occur during therapy. Supportive measures such as emollient therapy and wound care as well as trauma avoidance are also essential.

EBA is characterized by tissue-bound and circulating autoantibodies to type VII collagen, a unique basement membrane-specific collagen that is the major component of anchoring fibrils, located below the lamina densa of the BMZ. Type VII collagen is composed of 3 alpha chains, each composed of a 145 kDa central collagenous triple helical segment and flanked by 145 kDa amino-terminal noncollagenous (NC1) domain, and a small 34 kDa carboxyl terminal noncollagenous domain (NC2). Type VII collagen molecules form tail-to-tail dimers with disulfide bonding, and aggregate laterally to form anchoring fibrils with large globular NC1 domains at both ends of the adhesive fibrils. The NC1 domain is the major target antigen, composed of 4 immunodominant epitopes for EBA: cartilage matrix protein (CMP), fibronectin type III (FINIII)-like repeats, and von-Willebrand factor-a (VWF-A). Chen et al found that the NC1 domain facilitates binding with various extracellular matrix components including fibronectin, laminin-5, type I collagen and type IV collagen, which then serve to adhere BMZ to dermis. IB has shown patient IgG autoantibodies labeling a 290kDa protein, and reacting with the NC1, NC2 and triple helical domains of type VII collagen. Target antigen includes type VII collagen and laminin alpha-3 located in the sublamina densa level of blister formation. Blistering is thought to be due to interaction between epitopes on collagen VII and autoantibodies that form localized immune complexes. Fc domains of anti-type VII collagen antibodies trigger complement mediated inflammation, resulting in proteolytic degradation of matrix components within the dermal-epidermal junction (DEJ) essential for adherence, resulting in formation of dermolytic blisters. Another proposed mechanism includes binding of anti-type VII collagen autoantibodies to functional epitopes on the NC1 domain, interfering with interaction between this and other BMZ and papillary dermal components like type IV collagen, laminin-5, fibronectin, disturbing adhesive function. Passive transfer of autoantibodies against type VII collagen into mice induced Fc-dependent activation of complement, recruitment of leukocytes into skin and subsequent reproducible blister formation of EBA. Chen et al affinity purified anti-NC1 autoantibodies from EBA patients and injected into mice, inducing EBA-like subepidermal blisters. Antibodies have been
found to be IgG1 and IgG4 subclass predominant. These studies have proven that human EBA autoantibodies to the NC1 domain of type VII collagen are pathogenic and capable of inducing subepidermal blisters.

DIF reveals broad linear BMZ staining with immunoreactants, with IgG reacting more strongly than C3. IIF may be positive in 25-75% of cases but may also be repeatedly negative. IIF will reveal circulating autoantibodies staining the BMZ of monkey esophagus. In vivo immunomapping will show deposition of IgG and blister formation will be seen to occur at the level of the anchoring fibrils in the epidermal sublamina densa zone. Salt split skin will demonstrate immunoreactants binding in a dermal pattern at the floor of the induced blister. Antigen mapping will locate both BP antigens, laminin and type IV collagen at the roof of the blister.

IB of dermal extracts show IgG autoantibodies staining a 290 kDa antigen, (which represents the alpha chain of type VII collagen) and/or a 145 kDa protein (which represents a globular domain of the alpha chain of type VII collagen) which are the non-collagenous domain of collagen type VII. From keratinocyte extracts, sera will reveal IgG reactivity to the unprocessed 200 kDa and processed 165 kDa forms of the α3 subunit of laminin 5 and to its minor 145 kDa fragment.

IEM for in-vivo bound IgG performed on frozen specimens with immunoperoxidase or immunogold labelling will show a cleavage level below the lamina densa, and IgG deposits attached to the lower lamina lucida, upper lamina densa, or dermal side of the basal lamina, suggesting that autoantibodies against laminin 5 and type VII collagen are deposited in vivo. Immunogold EM will also show IgG aggregations along anchoring fibrils in the sublamina densa zone and resulting in destruction. Occasionally, duplication of the BMZ with dissolution of the lamina densa may be seen.

Chen et al developed an ELISA with a eukaryotic recombinant protein that has been found to be highly specific and more sensitive than IB analysis for the detection of anti-type VII collagen IgG antibodies, likely because ELISA is performed under native non-denaturing conditions. Delbado et al used serum of a patient with suspected drug-induced EBA using an eukaryotic-expressed recombinant form of human type VII collagen consisting of the complete NC1 domain, which demonstrated that sera had circulating IgG against the NC1 domain of type VII collagen. Chen et al later produced GST fusion proteins encompassing complete fibronectin III (3 out of 4 independent epitopes) and von Willebrand factor A regions (1 of 4 epitopes) of the NC1 domain, which were recognized by EBA autoantibodies. These 4 epitopes however were not found to be pathogenic. They recently developed a recombinant fusion protein corresponding to the N-terminal amino acids of NC1 and homologous to CMP and analyzed this using immunoblot and ELISA. IB reveals a 50kDa band of CMP recognized by 22/32 EBA sera and 2/3 BSLE sera but not normal sera. They found that 26/32 (81%) EBA sera and 2/3 bullous SLE sera reacted with the CMP domain in both assays. These anti-CMP autoantibodies likewise induced clinical and histologic and immunopathologic features of EBA in hairless mice, demonstrating that EBA autoantibodies to the CMP subdomain of type VII collagen are pathogenic and play a key role in subepidermal blistering for EBA. They concluded that FINIII and VWF-A and CMP subdomains of NC1 of type VII collagen are all epitopes recognized by a majority of EBA autoantibodies, but CMP epitope is pathogenic and is a prominent immunodominant site.
3. Linear IgA Bullous Dermatosis (LAD)

LAD, the adult counterpart of chronic bullous disease of childhood, is an acquired autoimmune blistering disease that is usually idiopathic, related to systemic disease or may be drug induced.\(^4\) It usually presents in an age range of 45-60 years with tense vesicles or bullae in an arciform pattern on normal or urticarial skin.\(^2,5,50\) There may be mucous membrane involvement and scarring\(^2,12\) and usual sites affected are extremities and trunk.\(^50\) LAD is usually self-limiting, and may last from 2-7 years before resolving, but usually shows a good response to sulfones or systemic steroids.\(^2,50\)

Histopathology shows a subepidermal blister with a neutrophil rich infiltrate and eosinophils, and papillary dermal neutrophilic microabscesses.\(^50\)

Immunologic target antigens in LAD are thought to be heterogeneous and include BP180 (BPAg2), BP230 (BPAg1) and 285 kDa linear IgA disease antigen.\(^49\) Etiopathogenesis of LAD may be related to IgA autoantibodies reacting to fragments of BPAg2 (BP180) which correspond to either a 120 kDa protein (the soluble ectodomain of BP180) or 97 kDa protein (fragment of extracellular BP180).\(^6\)

DIF shows linear BMZ deposition with mainly IgA and occasionally C3 and IgG.\(^2,5,50\) IIF will show low titers of anti-BMZ IgA autoantibodies in 60-70% of patients.\(^2,50\) Salt split skin shows 1 of 3 staining patterns: epidermal (antigen in lamina lucida), dermal (antigen in low lamina lucida or subbasal lamina location) and combined (deposits on both roof and floor of blister), reflecting antigen heterogeneity, and different locations of antigen binding in this disease.\(^10\) IB will demonstrate the 97 kDa antigen, now thought to be a fragment of the extracellular portion of BPAg2 (180kDa) and which usually correlates to the epidermal pattern of staining in salt split skin studies.\(^6\) The presence of anti-290 kDa and anti-145 kDa autoantibodies usually relate to a dermal pattern of staining on salt split skin.\(^7\) IEM will also show 3 patterns of binding: IgA binding to either lamina lucida alone, both lamina lucida and sublamina densa or only in the sublamina densa.\(^10,50\)

IB assays for LAD and other IgA-mediated autoimmune bullous disease usually yields negative results, however Pas et al found that when the primary incubating temperature was increased to 37 degrees C, 10/11 sera revealed IgA bound to the 180 kDa BP180 antigen.\(^51\)

**DRUG INDUCED LINEAR IgA BULLOUS DERMATOSIS**

Drug induced LAD differs from idiopathic LAD in histopathologic findings, course of disease and response to treatment.\(^99\) Most commonly associated drug is vancomycin, and other inciting drugs include penicillins, cephalosporins, and captopril, phenytoin, diclofenac and piroxicam, atorvastatin and glibenclamide.\(^48,52\) Eruption starts within 7-14 days of starting offending drug, and resolves within 3 weeks of discontinuing the medicine. Clinically, lesions resemble erythema multiforme, toxic epidermal necrolysis or may be morbilliform. Histopathology is characterized by subepidermal blistering with a predominantly neutrophilic infiltrate, but differs from idiopathic LAD by presence of basal vacuolization or a lichenoid interface dermatitis. DIF will be identical to idiopathic LAD, showing linear deposition of IgA at the BMZ, with weak staining of C3 or IgG.\(^53\) Plunkett et al noted that in patients with DIF showing purely IgA BMZ deposition (without IgG) the likelihood of drug-induced LAD was high, and that possibly up to 2/3 of all LAD cases were drug induced.\(^32\) IIF may rarely show intercellular IgA deposition\(^57\) or be negative. Immunoprecipitation has shown circulating antibodies targeting 210 kDa, 130 kDa and 83 kDa target anti-
ELISA studies to BPAG1 and BPAG2 may be likewise be negative. Other patients may show immunoblot and IEM microscopy findings consistent with an immune response to BP-associated antigens. It is speculated that the drug acts as a hapten, or modifies the antigenicity of structural proteins, triggering an immune response that affects epidermal BMZ structures. Other theories on the pathogenesis of drug-induced LAD has been suggested to be due to: drug induced T-cell activation, increased number of CD8+ T cells, increased levels of IL-5 resulting in increased IgA expression, and other T cell derived cytokines. Dapsone and steroid therapy may hasten resolution but are not necessary for spontaneous resolution.

B. BASEMENT MEMBRANE ZONE STAINING: GRANULAR DEPOSITION

1. Systemic Lupus Erythematosus

Lupus erythematosus (LE) is the prototypic autoimmune disease characterized by antibodies targeted against native antigens and a highly variable clinical presentation. Patients are usually reproductive age women who present with skin rash (malar and discoid), arthralgia, oral ulcers, photosensitivity, anemia, serositis, nephritis and neural disease, as well as distinct serological abnormalities, which are among the criteria for diagnosis recommended by the American College of Rheumatology. These criteria have shown a diagnostic sensitivity of 88% and specificity of 95% for SLE. LE is commonly classified into the systemic (acute) LE, subacute cutaneous LE, and discoid (chronic cutaneous) LE.

Patients with systemic LE (SLE) may present with skin manifestations, such as a malar or photodistributed erythema with fine scaling, vasculopathic lesions like purpura, digital ulcers and urticarial vasculitis. Histopathology will include vacuolar interface change with lymphocytes along the dermoepidermal junction, colloid bodies and dermal mucinosis. The LE cell may be seen as rounded histiocytes containing nuclear debris near the DEJ.

Subacute cutaneous LE (SCLE) presents usually with a photodistribution eruption of annular erythematous plaques, aggravated by UV exposure, viral infections, changes in serum calcium and hormonal changes. Histopathology includes a combination of vacuolar interface change with focal lichenoid zones, more prominent epidermal atrophy, scattered dyskeratotic keratinocytes, more pronounced follicular plugging and BMZ thickening, and dermal mucin.

Discoid (chronic cutaneous) LE (CDLE) may also present with a photodistributed rash on the seborrheic areas of the head and neck, however clinical lesions are erythematous papules and plaques with thick scale, central atrophy, follicular plugging with patulous openings and usually resolve with scarring and a hyperpigmented border. Histopathology will be more distinct and show prominent follicular plugging, alternating epidermal acanthosis and atrophy, interface dermatitis of the hair follicles, BMZ thickening, melanin incontinence, a deeper more intense periadnexal infiltrate, and mucin deposition.

The diagnosis of LE and its subtypes requires a combination of clinical, histopathologic and immunopathologic criteria, as each test contributes to a final diagnosis. Many factors such as location of biopsy, sun-exposure, topical steroid therapy, and duration of lesion may lead to differing positivity rates for DIF, making serologic tests such as anti-DNA and other tests more important in the diagnosis of lupus. Williams et al recommend that DIF testing of both lesional and non-lesional skin be done in cases of possible LE, since most results will be only “highly suggestive” of SLE (but not CDLE) in the
absence of positive serology.\textsuperscript{56} Reported sensitivities of DIF for LE have ranged from 58-93%.\textsuperscript{57}

**LUPUS BAND TEST (LBT)**

The LBT is defined as continuous broad linear to granular deposition at the DEJ of usually IgM, then IgG, C3, and/or IgA, or multiple reactants on non-lesional sun-exposed skin in a patient with SLE. However, currently it may refer to DIF of a lesion taken either on lesional or non-lesional skin, on sun-exposed or sun-protected sites. Sensitivity is 73\% in SLE patients, and specificity is 64\%, with a positive predictive value of 57\%.\textsuperscript{56} Strongest deposition is usually with IgM (90\%), followed by IgG. (Fig. 2) Williams et al found that IgM and/or C3 deposition was consistent with the diagnosis of SLE, along with serologic findings of an ANA titer of 1:64, decreased C3 or C4, and positive anti-Ro/SSA or anti-La (SSB) antibody. IgG, IgM, IgA and C3 deposition at the BMZ was consistent with SCLE when taken from lesional and non-lesional skin, along with a positive ANA titer above 1:64. Finding IgM, IgA, C3 deposition on BMZ alone or in any combination on lesional skin only was consistent with the diagnosis of CDLE.\textsuperscript{57} Although some authors suggest that IgM DEJ deposition is non-specific, others believe that intermittent IgM (and sometimes IgG, and more specifically IgA) DEJ deposition on sun-protected skin is enough to warrant a diagnosis of positive LBT, since no false positives are expected.\textsuperscript{54} Crowson et al recommend that initial biopsy for LBT should be taken on sun-exposed lesional skin to avoid false-negatives, and after diagnosis of LE is made, biopsy of non-lesional skin could be done to sub-classify and prognosticate disease course. Biopsy is usually taken either from sun-exposed normal skin on extensor forearm, upper arm or shoulder (with a positive LBT in 77\% of SLE cases), or normal sun-protected skin on volar forearm or buttck skin (positive LBT in 33-50\% of active SLE cases). In sun-exposed non-lesional skin, LBT is positive in 70-80\% of SLE patients, and negative in DLE and SCLE patients. This correlates with the presence of anti-dsDNA antibodies, severe clinical disease (in 55\% of SLE cases) and with renal involvement in 70\% of cases.\textsuperscript{54}

In CDLE, the most common findings are C3 and immunoglobulin deposits at the BMZ in both lesional and non-lesional skin, however many variables may affect positivity: lesions from sun-exposed areas have a higher positivity rate of 82\% over trunk biopsies, and short duration of the lesion and topical steroid treatment will also reduce DIF positivity.\textsuperscript{56} In the 9-45\% of LE patients with oral lesions (erythematous plaques, erosive or bullous lesions) with a lichenoid infiltrate on histopathology, DIF will be frequently positive and show linear or granular BMZ staining of IgM or IgM with or without C3 deposits as well as IgM deposition on cytoid bodies.\textsuperscript{56}

The supramolecular membrane attack complex of complement C\textsubscript{5a}-9 assay is an adjunct test to the LBT in the diagnosis of LE and other connective tissue diseases, and is performed using monoclonal anti- C\textsubscript{5a}, mouse antihuman antibody against patient skin as substrate. Epitopes on activated C9 of the terminal complement complex are fractions of C5b, C6, C7, C8, and C9 which are the main target of this indirect IF technique. Granular BMZ deposition of C\textsubscript{5a} is seen in 80\% in lesional skin of SLE cases, in 66\% of SCLE patients and in 60\% of DLE cases.\textsuperscript{54}

**SEROLOGIC TESTING IN LUPUS ERYTHEMATOSUS**

Serologic testing for antibodies help to confirm the diagnosis of LE when considered with clinical, histologic, immunofluorescence findings. Coverage of all the details on new
serologic assays are beyond the scope of this article but may be found in several excellent reviews.\textsuperscript{54, 59} The autoantibodies that are detected by serological tests in LE are directed against self-antigens found in cellular components like intracellular antigens on the cell nucleus (double and single stranded DNA), histones, and extractable nuclear antigens (ENAs).\textsuperscript{59, 60} Immunopathologic techniques include radioimmunoassay, immunoelectrophoresis, radial immunodiffusion (which detects high affinity Abs) and IF (which detects moderate and high affinity Abs), although the ELISA is predominating due to ability to detect both high and low affinity Abs, and quantify antibody titers, among many advantages. Disadvantage of the ELISA remains to be low specificity, requiring careful interpretation of results.\textsuperscript{59, 60}

**ANTI-NUCLEAR ANTIBODY (ANA) STAINING**

Screening testing for LE is done using either the fluorescent ANA test or an ELISA test. The fluorescent ANA test is an IIF using either mouse kidney or rat liver, or human Hep-2 cells (derived from cultured esophageal squamous cell carcinoma cells), which have been found to be more sensitive. Patterns of fluorescence may reflect the specific antinuclear antibody: 1) Homogeneous pattern associated with anti-histone\textsuperscript{59}; 2) Nuclear membrane staining (peripheral or rim pattern) reflects anti-DNA antibodies or anti-laminin antibodies found in SLE; 3) Fine speckled nuclear pattern reflects antibodies to extractable nuclear antigens including Ro/SSA, La/SSB, U1RNP and Scl-70; 4) Coarse speckled pattern correlates with anticentromere antibodies seen in scleroderma.\textsuperscript{59}

ANA testing for SLE has very high sensitivity and high negative predictive value (100%). Positive predictive value is however only 11%. Mutasim \textit{et al} recommends a titer of 1:160 or more to be clinically significant, since many false positives may be seen in people without connective tissue disease (such as elderly people, pregnant women, people with chronic infections or on certain medications like hydralazine), as well as correlating ANA results closely with clinical features.\textsuperscript{59} Screening ELISA for ANA utilizes tissue extracts containing nuclear components or recombinant molecules. ELISA results correlate with fluorescent ANA test results in 87-95% of cases. Sensitivity of ANA ELISA is between 69-98% and specificity is between 81-98%.

**Anti-dsDNA (native or double-stranded DNA) antibodies** are characteristic of SLE and are found in 50-83% of SLE patients.\textsuperscript{59} Anti-dsDNA titers usually correlate to presence of the lupus band on normal skin, low complement levels and lupus nephritis as well as a poor prognosis.\textsuperscript{59} Anti-dsDNA may be detected using IF of \textit{Crithidia luciliae}, a hemoflagellate organisms with a kinetoplast containing nDNA, however the ELISA is a more sensitive test and uses calf thymus extract.\textsuperscript{59} Nossent recommends screening with the sensitive ELISA,\textsuperscript{55} then following up positive results with more stringent assays using \textit{Crithidia luciliae}, Farr assay with circular dsDNA antigen (method of choice for some),\textsuperscript{61} ELiA anti-dsDNA assays or solution-phase ELISA to differentiate between “benign” LE with non-pathogenic anti-dsDNA antibodies versus “malignant” LE with renal disease, associated with pathogenic antibodies that may mediate end-organ dysfunction.\textsuperscript{55} Use of Farr assay or ELISA for dsDNA is also useful for monitoring LE patients, especially those with nephritis.\textsuperscript{51}

**Anti-histone antibodies** are characteristic of drug-induced SLE, being found in 90% of patients, but may also be found in 30% of patients with idiopathic SLE. IF using rat liver, complement fixation, RIA and commercially available ELISA kits may be used.\textsuperscript{59}
Antibodies directed to small ribonucleoproteins (sRNP) include anti-Ro (SSA) and La (SSB) and U1RNP. These are molecules that contain RNA and have enzymatic activity in RNA processing. These are detected using RIA techniques with low sensitivity but high specificity, and ELISA with a high sensitivity and low specificity. Anti-Ro (SSA) and anti-La (SSB) antibodies are considered a heterogeneous antigenic complex comprised of 3 different proteins (52 kDa Ro, 60 kDa Ro and La) and 4 small RNA particles. Anti-Ro (SSA) are characteristic of LE (in particular SCLE and neonatal LE) and Sjögren’s syndrome. In LE, presence of anti-Ro antibodies correlates with photosensitivity in patients with SCLE, as well as vasculitis. Anti-La(SSB) antibodies are usually found with anti-Ro(SSA) antibodies. Testing for both may be done in LE suspect patients with a negative ANA screening test. The RNA precipitation assay has the highest sensitivity and specificity, while counterimmunoelectrophoresis is considered the most reliable assay performing better than IB and some ELISAs with a sensitivity of 89% and specificity of 100%. The ELISA has a high sensitivity but low clinical specificity, giving false positive results. It is recommended to request anti-Ro/La only in clinically significant patients.

Anti-Sm antibodies are diagnostic of SLE and along with anti-dsDNA antibodies are included in the diagnostic ACA criteria, despite being found in only 5-40% of SLE patients. These may be associated with severity and activity of renal involvement. Many assays are used for the detection of Sm antibodies, including double immunodiffusion, IB, IP, ELISA, protein microarrays, and addressable laser bead immunoassay using purified or recombinant proteins and synthetic peptides. These autoantibodies are directed to the Sm antigen, which is a component of small nuclear ribonucleoproteins.

The Sm antigen is composed of 9 different polypeptides, and core proteins like the B/B', D1 and D3 polypeptides are the main target epitopes. SmBB and U1RNP share a cross-reactive epitope motif, and so Sm D is considered the most SLE-specific antigen. Recombinant SmBB from bacteria or insect cells is usually used in commercial ELISA kits. A more highly specific peptide called SmD3 (consisting of only 16 amino acids and dimethylarginine) has been used in the development of an ELISA. It showed a sensitivity between 10-12% and specificity of 88-100% in SLE but had the main advantage of being able to differentiate SLE from MCTD patients.

Anti-U1RNP antibodies are found in 100% of patients with MCTD, and in 25-47% of patients with SLE, and its presence is associated clinically with sclerodactyly, Raynaud’s phenomenon, pulmonary dysfunction and milder renal involvement. Anti-RNP antibodies react with proteins (70 kD, A, C) associated with U1RNP. Assays used to detect anti-RNP Abs are counterimmunoelectrophoresis, IB, and ELISA based on purified or recombinant proteins or synthetic peptides.

Anti-cardiolipin (anti-phospholipid, APA) antibodies are directed against phospholipids in cell membranes. These are associated clinically with livedo reticularis, purpura and necrosis, recurrent miscarriages, arteriovenous thrombosis, and thrombocytopenia. Because these antibodies had in vitro anticoagulant properties, they were labeled as “lupus anticoagulant”. This assay had a sensitivity of 75%. Currently, ELISA using bovine cardiolipin has a sensitivity of 90% for the 50% in SLE patients with APA, and may be used for screening.

Newer assays like anti-C1q and antinucleosome antibodies have been proposed for diagnosis and monitoring of SLE patients, along with urinary levels of monocye...
a. BULLOUS SYSTEMIC LUPUS ERYTHEMATOSUS

Bullous SLE is a rare chronic subtype of LE, diagnosed in less than 5% of patients with confirmed SLE. Diagnostic criteria were proposed by Camisa and Sharma and include: diagnosis of SLE by ACR criteria; vesicles and blisters on sun-exposed skin; histopathology compatible with DH; IIF negative for circulating BMZ antibodies; deposition of IgG and/or IgM and IgA at BMZ by DIF. Currently, diagnosis rests on the clinical findings of a pruritic generalized non-scarring tense vesiculobullous eruption, subepidermal vesiculobullous dermatitis with neutrophilic predominant infiltrate combined with interface dermatitis and dermal mucinosis, linear or granular IgG and/or IgM on DIF, and salt split skin IF showing autoantibodies to type VII collagen binding to the floor of the blister. Mainstay of treatment is dapsone, and alternatives include methotrexate, azathioprine, antimalarials and cyclophosphamide. Disease may remit in months to years.

Target antigens in bullous SLE may be heterogeneous, however the main antigen targeted by IgG autoantibodies has been found to be the non-collagenous (NC1) domain of type VII collagen (composed of sequential fibronectin type III homology units and an area homologous to the A2 domain of von Willebrand factor), identical to the target antigen in EBA. Type VII collagen is an epithelial BMZ restricted protein composed of 3 identical alpha chains (each consisting of an amino-terminal 145 kDa noncollagenous (NC1) domain and a 145 kDa collagenous domain). The NC1 domain contributes to BMZ-dermal adhesion by cross-linking lamina densa to type IV collagen, and cross-links dermis to dermal matrix proteins. The epitopes within the NC1 domain recognized by autoantibodies in bullous SLE are the FN-III homology units. IB will show IgG binding to 2 bands: 290kDa and 145 kDa, both specific for type VII collagen. Antibodies to type VII collagen activate complement cascade and generate C5, which is a neutrophil chemoattractant. Likewise, autoantibodies interfere with the function of type VII collagen by binding to proteolytic fragments containing the NC1 domain. Shirahama found that patients serum bound to multiple epitopes on the NC1 domain, located within the 1080 AA sequence encoded by fusion proteins FP1-6 by epitope mapping. Chan et al found a patient whose autoantibodies recognized 4 different BMZ components: BPAG1, laminin-5, laminin-6, and type VII collagen using IB (using culture conditioned medium for recombinant NC1 domain of type VII collagen, and heterotrimers of laminin-5/6 from human keratinocyte culture), immunoprecipitation (using human KC cultures containing laminin 5/6) and ELISA (using purified recombinant human type VII collagen NC1 domain) techniques. Multiple autoantibodies targeting different antigens are thought to be due to “epitope-spreading phenomenon” (primary autoimmune inflammation causes tissue injury, releasing sequestered antigenic epitopes, and leading to exposure of “new” antigenic epitopes to antigen-presenting cells and activation of autoreactive T and B cells, leading to a secondary autoimmune response and subepidermal blister formation.

DIF will reveal a broad linear, granular or pseudolinear (homogenous with superimposed granular band) pattern of IgG (+/- IgA) deposition along the BMZ. Occasional vessel staining with IgA, IgM and C3 may be found. Both kappa and lambda light chains may be found, and IgG subclasses may range from IgG1-4. Cytoid bodies will be seen in 29 of 117 specimens, stained by 3 or more immunoreactants, ranging from few to nu-
merous. Perifollicular and follicular cytoid bodies are seen in DLE but rarely in SLE.75 IIF may demonstrate circulating antibodies to type VII collagen in some, but not all patients.65,66 Salt split skin will reveal antibody staining usually the dermal side of the split (though occasional epidermal staining may be seen), and IEM will confirm colocalization of immunoreactants to the sublamina densa region (similar to EBA).57 Vodegel et al found that in EBA and bullous SLE, the “u-serrated” staining pattern was seen, corresponding to the ultrastructural location of type VII collagen in the sublamina densa zone.54

ELISA testing using purified recombinant human type VII collagen NC1 domain may be used in bullous SLE.27 Gandhi et al developed an ELISA to detect anti-type VII collagen autoantibodies in patients with both bullous SLE and EBA, using 4 eukaryotic recombinant proteins (1 full-length and 3 truncated noncollagenous domain 1 proteins). Majority of patient sera contained complement-activating IgG autoantibodies. Sera reacted to the N-terminal region of the noncollagenous domain 1 (thought to be the major epitope target), as well as cartilage matrix protein and fibronectin-like repeats. These anti-type VII collagen autoantibodies were polyclonal, containing kappa and lambda light chains, were concluded to be pathogenic and acted through complement-mediated injury and mechanical disruption of the anchoring function of type VII collagen.54 Alahlafi et al showed that there was colocalization of the main components of the skin BMZ using both DIF and confocal laser scanning microscopy with type VII collagen, however when patients sera was reacted with an ELISA using complete recombinant human NC1 domain of type VII collagen, none were found to be reactive, ruling out that this is the target antigen for circulating antibodies in patients with SLE and a positive LBT.69

b. DRUG INDUCED LUPUS ERYTHEMATOSUS

Drug induced lupus should be considered in patients who suddenly develop positive ANA, anti-histone antibodies and clinical features of LE while taking a drug, and which then resolves upon drug discontinuation. Histopathology and DIF findings would be very similar to idiopathic LE. Most common drugs causing drug induced LE are hydrochlorothiazide, angiotensin-converting enzyme inhibitors, terbinafine,70 chlorpromazine, hydralazine, procainamide and quinidine, and methyldopa.54 Some of these drugs inhibit T cell DNA methylation, depressing T-suppressor lymphocytes activity, and inducing autoimmunity in T-helper lymphocytes and inducing B-cell differentiation.54 SCLE may be triggered in elderly patients by drugs such as thiazides and calcium channel blockers (which decrease calcium concentration in keratinocyte and lymphocyte cytosol, displacing Ro/SSA antigen onto cell surface, promoting antibody binding and antibody-dependent cellular cytolysis).54

Fig. 2
Broad granular staining of IgM at the basement membrane zone reactions in a case of lupus erythematosus. (fluorescence microscopy, original magnification x400)
2. Dermatitis Herpetiformis

Dermatitis herpetiformis (Duhring’s disease, DH) is an autoimmune blistering disease characterized clinically by a symmetric pruritic vesicular eruption associated with an immune-mediated gluten-sensitive enteropathy or celiac disease. The current suggested criteria for diagnosis includes: 1) typical clinical appearance of DH, and histopathology revealing subepidermal vesiculobullous dermatitis with neutrophil predominance; 2) positive DIF showing granular IgA deposition at BMZ and papillary dermal tips; 3) one or both serum tests to detect autoantigen tissue transglutaminase (tTG), either the anti-endomysial antibody IIF (AEma) or ELISA test to detect tTG particularly in patients with an initially negative DIF. Any 2 of 3 criteria are consistent with the diagnosis of DH.

Recent studies have identified the reactive antigen in DH as being tTG. Patients produce antibodies to tTG-gliadin complexes, and clinical DH results from a failure of the tTGS to metabolize gliadin and their glutenin polymers (consumed in wheat, rye and barley products). IgA antiendomysial antibodies play a pathogenic role, as revealed by its high sensitivity and specificity to DH and celiac disease, and by formation of these antibodies in response to gluten response challenge and in vitro bindings of gliadin to antigen sites of these antiendomysial antibodies.

DIF reveals a granular deposit of IgA at the BMZ, with a concentration at papillary dermal tips. (Fig. 3) DIF has been found to have sensitivity for DH of approximately 90%. While DIF remains the most definitive diagnostic test for DH, up to 10% of DH patients may have negative DIF studies.

Chorzelski et al developed an IIF method to detect IgA AEma to diagnose DH, which has been included in the diagnostic criteria for celiac disease and gluten sensitive enteropathy. The lower half of monkey esophagus is incubated with patient serum and stained with fluorescein-conjugated IgA to detect IgA endomysial antibodies, resulting in a reticular staining pattern around nonreactive smooth muscle myofibrils in the connective tissue of the smooth muscle layer subjacent to the BMZ. Overall sensitivity of AEma for diagnosis DH was 79% and specificity is 96%. For DH patients not faithfully following a gluten-free diet regimen, sensitivity of AEma IIF increases from between 60-90% but may it be cheaper and more specific than tTG ELISA.

The identification of tissue transglutaminase that binds AEma in IIF tests as the target antigen led to the development of a tTG-ELISA, which has been gauged to be more than 99% specific for DH and/or celiac disease. The advantages of this ELISA are that it is a rapid objective, minimally invasive, cost effective and quantitative test to detect anti-tTG antibodies. Recent studies comparing AEma IIF with tTG-ELISA revealed agreement in about 90% of cases both quantitatively and qualitatively. Currently, it is agreed that the ELISA to tTG antibodies has a comparable sensitivity to DIF for DH.

Fig. 3
Granular staining of the basement membrane zone with IgA with accentuation at papillary dermal tips in a case of dermatitis herpetiformis. (fluorescence microscopy, original magnification x400)
and a higher sensitivity than then AEmA IIF for gluten sensitivity, with a sensitivity of 94.4%. Beutner found specificity for the ELISA to be lower than the AEmA IIF with false positive results however other studies cited a specificity of 92.3-100%. Because DH cases may give weak or no AEmA or tTG-ELISA reactions, Beutner et al recommends that both methods be used simultaneously.

The tTG-ELISA reveals that anti-tTG antibodies decrease to normal in patients on strict gluten-free diets, and is an effective way to monitor compliance and response to therapy. Likewise, in patients with negative DIF and positive ELISA tests, to rule out false positive results, Beutner et al recommend a gluten-free diet and a gluten challenge to monitor clinical responses. Currently, the tTG-ELISA is recommended as first line screening serological test for celiac disease. Desai et al recommend using the ELISA as a practical first step in the diagnosis of DH.

3. Drug eruptions

Drug hypersensitivity reactions are usually diagnosed with the occurrence of a rash after initiation of drug therapy which resolves after discontinuation of the medication. Incidence of drug eruptions is about 2.2% and usually occurs more in females and inpatients. In general, the most common culprits are antibiotics, followed by NSAIDs, anti-epileptics, anti-tuberculous and cardiac medications. Mechanisms of drug hypersensitivity are unknown, but may be related to high doses or prolonged duration of therapy, a higher risk with increased number of drugs taken simultaneously, immunodeficiencies (such as in childhood, geriatric and HIV population), the slow acetylation phenotype, glutathione deficiency and coexisting viral infections or immune complex deposition. Immunologically, type I IgE-mediated immediate-hypersensitivity reactions will present clinically with urticaria, angioedema, anaphylactic reactions. Type III immune-complex mediated eruptions will present with serum-sickness like manifestations, vasculitis or the Arthus phenomenon. Type IV cell-mediated cutaneous drug eruptions will present usually with lichenoid eruptions, LE-like syndromes, fixed drug eruption or erythema multiforme, Stevens-Johnson syndrome or toxic epidermal necrolysis. Clinically, drug eruptions are protean and may manifest commonly as a morbilliform or maculopapular eruption, purpura and acneiform eruptions, erythema multiforme, generalized exfoliation, blistering, lichenoid lesions, pustular lesions and so on. Histologic features either reflect phenotype of the clinical lesion (neutrophilic spongiosis in purpuric eruptions, leukocytoclasis in purpuric lesions), however the common morbilliform eruption on biopsy will show spongiosis, basal vacuolization, dyskeratotic keratinocytes and a mild to moderate dermal perivascular infiltrate with lymphocytes, histiocytes and few eosinophils. Immunohistochemistry will reveal a predominantly CD8+ activated T cell lymphocytic infiltrate, and increase in epidermal proinflammatory cytokines like TNF-α, IL-1 and II-6. DIF will be rarely positive, and may show granular C3, fibrinogen, IgM, IgG or IgA deposition at the BMZ and within dermal blood vessels.

4. Erythema Multiforme

Erythema multiforme (EM) is characterized by recurrent targetoid lesions and vesicles affecting skin and rarely one mucosal surface. It is usually associated with herpesvirus infection flares and rarely caused by drug intake. Diagnosis is made clinically, and histopathology will reveal an interface dermatitis with dyskeratotic keratinocytes, vacuolar interface change and focal epidermal necrosis.
DIF is positive in about 88% of EM patients. Most common IF findings are cytoid bodies found in 45-67% of cases, correlating to intraepidermal or subepidermal necrotic keratinocytes, and granular deposition of C3, IgM, fibrinogen and IgA at upper dermal blood vessels. Other IF patterns include: linear deposition of IgG or fibrinogen at the BMZ, granular BMZ staining with C3 or properdin, epidermal nuclear fluorescence (homogenous staining with IgM or speckled staining with C3), and intercellular C3 staining. Finan et al concluded that HSV-associated EM showed a mixed DIF pattern (epidermal changes plus dermoepidermal junction deposits) whereas drug-related EM showed mostly epidermal changes on DIF. Patient sera tested using traditional IF are usually negative, however circulating immune complexes may be detected using monoclonal rheumatoid factor inhibition assays.

IB using epidermal extracts has demonstrated circulating antibodies reacting to BP230 (BPAg1). It has been attributed to a type IV delayed type hypersensitivity reaction, but recent studies have shown autoantibodies directed against desmoplakin I and II in a subset of patients with severe EM. Using IB, immunoprecipitation and epitope mapping, EM patients’ autoantibodies recognized an epitope identified as YSYSYS, which regulates keratin filament assembly in desmosomal plaques.

A highly specific and sensitive ELISA was developed by Hinterhuber et al using a synthetic peptide containing amino acid sequence found within the carboxy terminal domain of desmoplakin, which found all EM patient sera to be positive and only 1 sample from control sera.

5. False positives (rosacea, drug eruption, leprosy, sun damage, others)

DIF of biopsies from sun-exposed skin of 20-25% of normal healthy adults without history of systemic disease, previous dermatosis or photosensitivity, and those with actinic keratoses and rosacea may reveal falsely positive results, seen as weak linear or intermittent granular IgM, IgG, IgA and C1q deposition at the BMZ. Nieboer biopsied both sun-exposed and protected skin from 25 healthy volunteers, and found that all biopsies had C3 deposition at the BMZ seen as fibrillar, interrupted granular staining, while sun-exposed skin had additional weak linear BMZ staining with IgG, IgM, albumin and fibrinogen. Kulhanan found weak focal IgM deposition at the DEJ in 42.5-59% of 200 patients with non-LE dermatoses, while Helm saw continuous linear deposition of only IgM at the BMZ in absence of deposition of other immunoreactants in patients with urticaria, hypersensitivity dermatitis and leukocytoclastic vasculitis, suggesting that a positive lupus band should only be diagnosed when strong intense deposition of IgM in combination with other immunoreactants is seen. Caro et al found granular or linear deposition of only IgG at the BMZ in 52.8% of 36 patients with primary fibrositis syndrome and in 16.7% of healthy controls. Fibrinogen may commonly be seen in normal adults as a bright intense continuous granular deposition at the BMZ and therefore interpretation of biopsies from sun-exposed skin may be non-specific and should be done with caution. Vascular deposition with C3 or IgM may be seen in normal sun exposed and protected skin, as well as granular deposition of C3, IgM, C5 and properdin within arrector pili muscles. Granular or linear C3 or IgG deposition at adnexal BMZ may also be seen in normal skin.

C. BASEMENT MEMBRANE ZONE STAINING: SHAGGY FIBRINOGEN
DEPOSITION

1. LICHEN PLANUS

Lichen planus (LP) is a chronic autoimmune disease affecting skin and oral and genital mucosa, and is characterized clinically by pruritic violaceous flat-topped papules with white striations (Wickham’s striae). On the oral mucosa, LP may present with painful white papules and plaques, Wickhams’ striae, erosions or blisters, affecting most frequently buccal mucosa, then tongue dorsum and gingival surfaces. The pathogenesis of LP is still unclear, but it has been associated with systemic medications like anti-malarials, dental amalgams, hepatitis C viral infection, and tobacco chewing. Histopathology is characteristic and includes orthohyperkeratosis, wedge-shaped hypergranulosis, irregular saw-toothed acanthosis, and a dense bandlike infiltrate of lymphocytes closely apposed to the epidermis, many dyskeratotic keratinocytes (colloid or civatte bodies, ultrastructurally found to be apoptotic keratinocytes) and diffuse vacuolar interface change.

Lichen planus is recognized to be a T cell-mediated immune disease, where T cells are activated by recognition of lichen planus specific antigen (LPSA) associated with major histocompatibility class I on basal keratinocytes, and release of cytokines leading to chronic inflammation. CD8+ cytotoxic T cells release TNF-α, triggering the caspase cascade resulting in keratinocyte apoptosis. Lichen planus specific antigen is still undefined, and is speculated to be any of the following: an autoreactive peptide, exogenous proteins like drugs, contactants, viral or infectious agents. Humoral immunity likewise plays a role, since circulating antibodies have been found against various antigens like desmoglein 1 and 3.

DIF staining pattern in LP is highly characteristic but not specific, revealing a broad linear “shaggy” deposition predominantly of fibrinogen, with deposition on cytoid bodies of IgM (most frequently, followed by fibrinogen, IgA, C3 and IgG). Sensitivity of DIF for oral LP lesions has been found to be about 66%, with rate of positivity increasing with increased number of biopsies (72% in 1 biopsy versus 100% with 4 mucosal biopsies), punch technique and specific sites like mouth floor and ventral tongue. Early indirect immunofluorescence studies using autologous human skin or allogenic lesional tissue from LP patients detected circulating antibodies directed against a lichen planus specific antigen found only in the stratum granulosum and upper spinosum in 80-88% of LP patients. They found this test to be specific to LP, and suggested its use in differentiating between LP, atypical cases and LE, which may have similar histologic and DIF findings. Also, IIF using rat esophagus detected antibodies against the cytoplasm or membrane of basal cells. IIF have been done to detect anti-nuclear antibodies to stratified epithelia (SES-ANAs) which target an unknown antigen with MW 70kDa, a member of the p53 family which is strongly associated with chronic ulcerative stomatitis. Initial studies detected a

Fig. 4
Broad “shaggy” staining of the basement membrane zone reactions with fibrinogen in a case of lichen planus. (fluorescence microscopy, original magnification x400)
speckled IgG antibody deposition pattern on keratinocyte nuclei in 40.4% of LP patients using rat esophagus, and 27.6% using monkey esophagus. Parodi et al used IIF on 3 substrates: HEp2-2000 cells, normal human skin and monkey esophagus. IB using cultured keratinocytes and normal human skin as antigen sources were likewise used. Of 138 LP patients, 13.8% had positive IIF to monkey esophagus, 15% IIF positive only to HEp2-2000 cells, and 34.7% positive to both substrates, verifying that they were SES-ANAs, of which 47% were directed against the 70kDa antigen by IB. Autoantibodies to adhesion molecules desmoglein 1 and 3 were tested in 57 patients with oral LP using a commercial ELISA kit to Dsg 1 and Dsg3. Patients with erosive LP were found to have significantly higher levels of anti-desmoglein autoantibodies than healthy controls, suggesting that anti-keratinocyte antibodies are involved in LP. Patients with oral erosive LP were likewise found by IIF to monkey esophagus and rat bladder to have significantly higher antibody levels to epithelial components. The authors expressed doubt that anti-desmoglein autoantibodies were primarily pathogenic, and likely resulted from non-specific injury and epitope spreading.

Etiopathogenesis of LP has been speculated to be due to both cell-mediated and humoral immunity. T-cell mediated immunity against basal keratinocytes is triggered by antigenic modification at skin and mucosa, migration through cell surface adhesion receptors and molecules mediate binding to keratinocytes and extracellular matrix component leading to cytokine formation and inflammation. P-selectin is a cell surface glycoproteins that is a member of the selectin family of cell surface adhesion molecules which translocates to cell surface after inflammatory trigger factors and mediates leukocyte rolling and T-cell migration. P-selectin levels are usually elevated in patients with Behcet’s disease, SLE, scleroderma and other autoimmune diseases. Teoman et al tested sera from LP patients using commercially available ELISA sandwich assay and found that P-selectin levels were significantly higher in LP sera than normal controls.

a. LICHEN PLANUS PEMPHIGOIDES

Lichen planus pemphigoides is a rare autoimmune bullous disease with clinical and histologic features of both LP and BP. Clinical lesions present as pruritic erythematous patches and LP like papules with central blister formation and tense bullae which appear on lichenoid lesions and on previously unaffected skin. Histopathology may show features of LP from lichenoid lesions and features of BP from blistered skin. Lichen planus pemphigoides probably represents an entity distinct from either BP or LP, as bullous lesions are preceded by papules and plaques, and has a better response to therapy.

DIF of lichen planus pemphigoides will show linear BMZ deposits of IgG and C3 in perilesional skin (differentiating it from bullous LP), and fibrillar fibrinogen deposits as well as cytoid bodies. IIF using human skin has shown circulating IgG autoantibodies directed against the epidermal side of salt split skin, similar to BP pattern of staining. In oral lesions, fluorescence overlay antigen mapping and laser scanning confocal microscopy shows colocalization of in situ antibodies with β4-integrin, found in upper lamina lucida in close proximity to BP antigens. IEM has also demonstrated antibody binding to the hemidesmosomes and lamina lucida. IB using epidermal extracts has shown lichen planus pemphigoides sera contains IgG autoantibodies against the NC16a domain of recombinant BPAg2 (180kDa) however, no reactivity against the COOH-terminal of BP230. A 200 kDa and a 240kDa antigen have also been demonstrated by IB.
In IB and ELISA studies, lichen planus pemphigoides sera has been shown to react with the major target antigen NC16a domain of BP180 (a clustered set of 4 epitopes within the N-terminal 45 amino acids of the NC16a domain of BP180, designated as MCW-0-1-2-3, and corresponding to regions 1 to 3 of NC16a). Zillikens et al developed an ELISA using purified recombinant GST-NC16a1-5 of BP180, and found that lichen planus pemphigoides sera reacted with a novel epitope located on region 4, a 14 amino acid protein segment (46-59, designated MCW-4) within the NC16a domain of BP180 (distinct from BP sera), as well as showing reactivity against the full-length domain. They concluded that IB and ELISA studies using recombinant BP180 NC16a as target antigen were more sensitive than IB using human epidermal extracts for detecting anti-BP180 autoantibodies in lichen planus pemphigoides.

b. LICHEN PLANOPILARIS

Lichen planopilaris is characterized by a scarring alopecia with patchy hair loss, perifollicular erythema and keratosis, with skin, mucous membrane and nail features of typical LP. Histopathology will show epidermal changes of LP as well as interface change of infundibulum and isthmus, with perifollicular lymphocytic infiltrate, thinning and concentric fibroplasia. DIF will show linear deposits of IgG, IgA and/or fibrinogen on hair follicle BMZ, reveal patchy or linear fibrinogen deposition at the BMZ, as well as IgM and IgA staining cytoid bodies.

II. VASCULAR STAINING

A. VASCULAR STAINING: GRANULAR DEPOSITION

1. Immune Complex Vasculitis

Leukocytoclastic vasculitis (LCV) is characterized clinically by palpable purpura on the lower extremities and histologically by vessel wall damage and inflammation, extravasated erythrocytes with neutrophilic infiltrate and nuclear dust, and fibrin deposition, usually affecting small post-capillary venules. When immune complexes are deposited on vessel walls, neutrophils adhere to endothelial cells and release lytic enzymes. Vasculitis may be primary, or be secondary, with a cause found in 67-72% of vasculitis patients including hypersensitivity to drugs, infections, chemicals or associated with connective tissue diseases or underlying malignancy. Clinical presentation is usually palpable purpura, but confluent purpura, cutaneous necrosis, ulcers, pustules and urticaria may be seen, affecting usually lower extremities or whole body. Associated symptoms include pruritus, arthralgia and abdominal pain. Fever and paresthesia have been found to be risk factors for systemic involvement, while painful skin lesions were found to have a protective influence. Laboratory tests may be non-specific and show high erythrocyte sedimentation rate, anemia, leukocytosis, eosinophilia, thrombocytosis, urine abnormalities, elevated urea and creatinine and transaminase levels. Systemic involvements of other organs apart from skin may be seen in 20% of patients. On histopathology, about 60% of biopsies will show small vessel vasculitis, 40% with medium sized vessel vasculitis, and a predominantly neutrophilic infiltrate. LCV has a duration of disease ranging from weeks to months, a variable prognosis (spontaneous resolution, chronicity or recurrence in untreated patients) and generally low mortality rate of less than 2%. Therapy with dapsone and/or steroids is usually effective.

Criteria for the diagnosis of hypersensitivity vasculitis includes age greater than 16 years, intake of medications at onset of disease, palpable purpura, maculopapular rash,
and biopsy showing LCV of both venules and arterioles. Henoch Schonlein purpura (HSP) is diagnosed clinically with a triad of joint pain, gastrointestinal symptoms and purpura in a young patient and confirmed by histologic leukocytoclasia and IgA vascular deposits on DIF. Wegener’s granulomatosis (WG) is a systemic vasculitis characterized by a granulomatous vasculitis with giant cells, airway involvement (pulmonary nodules, sinusitis, otitis media), and glomerulonephritis. Microscopic polyangiitis (MPA) is a systemic vasculitis with glomerulonephritis, and no airway symptoms, and cutaneous polyarteritis nodosa (arterial vasculitis). Churg-Strauss syndrome (CSS) is a systemic vasculitis with glomerulonephritis, giant cells and granulomatous vasculitis, with asthma and peripheral eosinophilia.

Etiopathogenesis of vasculitis is thought to be due to trigger antigens (like infectious agents) stimulating circulating IgA (the primary antibody found at mucosal boundaries like respiratory or gastrointestinal system as defense against microbial agents). Immune complexes deposit within the postcapillary venule walls and activate classical and alternative complement pathways which attract neutrophils that adhere to endothelial cells, phagocytize immune complexes, disintegrate and release lysozomal enzymes damaging vascular endothelium. In HSP, target antigens include cardiolipin (which with phosphatidylserine are anionic phospholipids that compose the inner cell membrane). Due to endothelial cell damage, cardiolipin, and its circulating binding co-factor β2-glycoprotein I (β2-GPI), bear antigenic epitopes which anti-cardiolipin antibodies (aCL Abs) bind to. These anti-cardiolipin antibodies may be of IgG, IgM or IgA classes.

DIF of LCV will show deposition of immunoglobulin, complement components and fibrin in and around blood vessel walls, with IgG more likely to be related to connective tissue disease, and IgA deposits associated with Henoch Schonlein purpura. (Fig. 5) DIF testing was positive in 84.3-97% of patients with LCV but may be negative in 16% of patients. DIF shows IgG, IgA, IgM and C3 granular deposition in vessel walls, with fibrinogen being the most frequently deposited. About 47% of patients will show immunoreactant deposition in superficial blood vessels, while 3% may have deposits in deep dermal vessels, and a combined pattern in 26% of LCV patients. About 39% of vasculitic lesions will also show immunoreactant deposition at the dermoeipidermal junction. In LCV, IgA is usually the most frequently detected immunoglobulin in a range of 12% to 82% in lesional skin and in 68% of perilesional skin of vasculitis patients. C3 has also been cited to be the most commonly deposited immunoreactant in 71-80% of cases, IgM (35-56% and 34%), IgG (8-20% and 8%) are the other frequently deposited immunoreactants. IgA deposits on DIF of were noted to have a sensitivity for the diagnosis of vasculitis in 82% of lesional skin and 68% of perilesional skin, and a specificity of 73% and 66.7% respectively. Other studies noted that vascular IgA deposits in conjunction

![Fig. 5](image_url)

Granular staining of dermal blood vessels with IgA in a case of Henoch-Schonlein purpura. (fluorescence microscopy, original magnification x400)
with IgG and IgM deposition (which may be seen in 78% of cases) had low specificity for Henoch-Schönlein disease, since this pattern may be seen in various other diseases like drug induced vasculitis, rheumatoid arthritis, chronic alcoholic liver or glomelular diseases, erythema nodosum, venous stasis, coagulopathies, cryoglobulinemia and livedoid vasculitis. Isolated IgA staining (+/- C3) in a distinct stippled pattern may be characteristic of HSP and is seen in up to 50-75% of clinically diagnosed HSP, however it not specific, and diagnosis of HSP should still rest on clinical criteria like gastrointestinal involvement, upper respiratory tract infection and age less than 20 years.

Biopsy of uninvolved skin in patients with HSP showed 78% with positive DIF showing vascular deposition, and 67% having IgA deposits, again suggesting that biopsy of uninvolved rather than lesional skin for DIF may be more helpful in confirming the diagnosis of HSP.

IgM and C3 will be the most common immunoreactants deposited in patients with cryoglobulinemia. In patients with WG, immunoreactants deposits will be C3, fibrinogen, IgG, IgM and IgA (with IgA staining in a pattern identical to HSP patients). Occasionally, vascular deposits of C3 and fibrinogen will be seen in non-lesional skin of WG patients.

With regards to timing of biopsy for DIF, early lesions of vasculitis (1 day or less) will show deposits of fibrinogen, C3 and IgM in 82% of cases, while fully developed lesions taken between 2-7 days will usually demonstrate fibrinogen and C3 vessel wall deposits in 74% of cases, with an overall positive DIF yield of 76% in biopsies performed within 1 week of onset of LCV. Sais et al found that positivity of DIF test was inversely correlated with duration of the lesion studied, since biopsies of lesions older than 48 hours will be frequently negative due to destruction and removal of immunoglobulins. Bagai et al found that immune deposits may still be found on DIF of lesions older than 24 hours to 5 days, although immune deposits become weaker with time due to degradation by neutrophils.

Since IgA-type anticardiolipin antibodies have been speculated to play a role in HSP, Kawakami et al developed 2 ELISA systems using highly purified cardiolipin to detect IgA autoantibodies in HSP patients. They found that all HSP patients had elevated levels of IgA anti-cardiolipin antibodies, but no IgG or IgM anti-cardiolipin antibodies. There was a clear association between IgA anti-cardiolipin antibodies and CRP levels, disease activity, presence of arthralgia and proteinuria, and they suggested that monitoring serum IgA anti-cardiolipin antibodies might be helpful to predict renal involvement. Anti-neutrophic cytoplasmic antibodies (ANCA) is a diagnostic test in idiopathic systemic vasculitides performed using IIF on ethanol-fixed neutrophils to discriminate between a cytoplasmic ANCA (c-ANCA, usually found in WG and perinuclear ANCA (p-ANCA, usually seen in microscopic polyangiitis (MPA). Overall sensitivity for the IIF for ANCA is between 81-85% with a low specificity of 76%, mostly due to presence of pANCA in SLE patients. The overall sensitivity of IIF to cANCA in WG is 66% with a specificity of 98%. Major target antigens for ANCA has been characterized as enzymes isolated from neutrophilic granules (proteinase-3(PR3) in cANCA and myeloperoxidase (MPO) enzymes in p-ANCA pattern). Since the IIF ANCA test has been found to be antigen non-specific, Hagen et al developed and compared a standardized anti-PR3 and anti-MPO ELISA, and tested sera from patients with WG, MPA and CSS. Sensitivity IIF for cANCA in WG patients was 64%, and pANCA 21%, with a combined sensitivity of 85%. Sensitivity of pANCA in MPA patients was 58% vs cANCA of
23%, with a combined sensitivity of 81% in MPA. Specificity for healthy controls for the IIF of both c- and p-ANCA was 94%. Specificity for the ELISA using anti-PR3 and anti-MPO was set at 90%. For WG, sensitivity of anti-PR3 ELISA was 67%, and 24% for anti-MPO ELISA. For MPA, 58% had anti-MPO antibodies and 27% had anti-PR3 antibodies, and very low numbers for both CSS and classic PAN. Specificity for the ELISA using anti-PR3 and anti-MPO was set at 90%. For WG, sensitivity of anti-PR3 ELISA was 67%, and 24% for anti-MPO ELISA. For MPA, 58% had anti-MPO antibodies and 27% had anti-PR3 antibodies, with very low numbers for both CSS and classic PAN. Specificity for anti-PR3 ELISA was approx 87%, and specificity of anti-MPO ELISA was 96%. Use of ELISA with purified antigens alone was not more sensitive than traditional IIF for ANCA. However, in comparison with sole usage of IIF test for ANCA detection, the combination of IIF with ELISAs for anti-PR3 and anti-MPO resulted in a 10% lower sensitivity, but an increase in specificity to 98%, and therefore, combining both assay methods (IIF ANCA and antigen specific ELISA) will optimize the diagnosis of idiopathic necrotizing small vessel vasculitis.

B. VASCULAR STAINING: HOMOGENEOUS DEPOSITION

1. Porphyrias

Porphyria cutanea tarda (PCT) is an acquired disorder of uroporphyrinogen decarboxylase, an abnormality in heme biosynthesis resulting in excretion of excessive amounts of urinary uroporphyrin levels. It is classified as a “hyalinizing vasculopathy” due to the hyaline-like thickening of dermal blood vessels. Exogenous factors in the acquired form of PCT include alcohol abuse, iron overload, estrogen intake and exposure to hexachlorobenzene, and hepatitis C virus infection. PCT is also associated with chronic liver disease, including steatosis, hemosiderosis, and eventual hepatocellular carcinoma. In iron overload, it is speculated that iron ions leads to free radical formation, inhibiting uroporphyrinogen decarboxylase liver activity. The accumulated uroporphyrin is activated by ultraviolet light at 400- to 410-nm spectrum, leading to complement activation. Pseudoporphyria is caused by drugs (naproxen, tetracycline, amiodarone, gold and some diuretics), hemodialysis and even UVA tanning salons, and shows clinical, histologic and immunopathologic features identical to acquired PCT but with normal porphyrin levels. Pseudoporphyria is thought to be due to complement activation triggered by underlying photosensitivity and attenuated by intake of an offending drug.

Clinically, PCT is characterized by photosensitivity, skin fragility, and tense hemorrhagic bullae that heal with scarring and milia formation. Histologically, characteristic features include non-inflammatory subepidermal blisters with papillary dermal festooning, dermal sclerosis, and characteristically homogeneous eosinophilic deposits of Periodic acid-Schiff (PAS) positive, diastase-resistant material around blood vessels in the dermal papillae. Diagnosis will rest on findings of abnormal porphyrin metabolism (increased levels of porphyrin metabolites) evaluated by high-pressure liquid chromatography with fluorometric detection or colorimetric determination to measure coproporphyrins, uroporphyrins, and intermediate porphyrins.

DIF of biopsies from patients with PCT will show thick, brightly fluorescent homogeneous or “waxy” deposits of immunoglobulins and complement (usually IgM and C3) within blood vessels in the upper and mid dermis. IgG and C3 (also, IgM and fibrinogen) may also be found deposited at the BMZ at the dermoepidermal junction, along with IgM-staining cytoid bodies. The combination of vascular staining plus BMZ fluorescence distinguishes PCT and pseudoporphyria from the pemphigoid group and EBA. (Fig. 6) These are diagnostic of PCT but are not specific, as they may be seen in pseudo-
porphyria as well, with 77% of cases showing blood vessel IgG deposition, and cytoid bodies in 31% of cases.\textsuperscript{107} Antigen mapping using BP antigen, type IV collagen and laminin were done in 5 PCT and 3 drug-induced pseudoporphyria patients, which showed BP antigen staining the roof of the blister and type IV collagen and laminin at the floor of the bulla, indicating the level of split at the lamina lucida for both.\textsuperscript{116} IB of PCT sera have shown autoantibodies targeting an unknown antigen with MW 40kDa, however this was interpreted to be a reaction to hepatitis C infection. Immunoelectron studies in PCT have shown reduplication of vascular and epidermal basement membrane and collagen fibrin deposition due to microvascular injury. Basement membrane traps immunoglobulins, complement and fibrinogen, which is seen as fibrillar and amorphous deposits on and around blood vessels.\textsuperscript{114, 115}

Antinuclear antibodies against liver antigens (50, 45 and 56kDa antigens) were detected but were of unknown pathologic significance.\textsuperscript{118} An antibody-dependent cell-mediated cytotoxicity test to detect serum antibodies against porphyric or normal rat hepatocytes was done in 10 PCT patients showing high cytotoxicity against porphyric hepatocytes and low reaction against normal hepatocytes. The presence of these antiporphyrinic hepatocytes antibodies indicates that hepatocellular porphyrin might be partially responsible for the antigenicity of liver cells, but pathogenicity is still undetermined.\textsuperscript{118}

IIF using anti-C5b-9 antibodies was performed by Vasil \textit{et al} to detect presence of C5b-9 membrane attack complex in skin of PCT patients. C5b-9 deposition in blood vessels is seen in immune-mediated microvascular injury due to complement activation via UV-activated uroporphyrins, antienothelial cell antibodies or other circulating immune complexes.\textsuperscript{115} They also used IIF to detect presence of C3d and C4d in PCT patients. They found intense granular deposition of C5b-9 within walls of blood vessels in the superficial to mid dermis in all 31 PCT cases, as well as granular and homogeneous C3d and C4d deposition in some of the cases. This study suggested that this assay is a helpful diagnostic adjunct and that C5b-9 is an effector mechanism of microvascular injury in PCT.\textsuperscript{115}

III. CYTOID BODIES

Cytoid bodies are usually dyskeratotic or necrotic keratinocytes within the epidermis or at the dermoepidermal junction that have been stained with immunoreactants. Cytoid bodies were found by DIF in 10.8% of 1080 cases, and 70% of specimens exhibited interface change like vacuolar change and cell necrosis. (Fig. 7) Cytoid bodies were found in LE (25% of 113 cases), EM (18.8%), LP, dermatomyositis, toxic epidermal necrolysis, LCV, BP, LP, and PLEVA. Most frequently deposited immunoreactant was IgG, followed by IgM (most brightly staining), C3, IgA and fibrinogen. Staining is non-immunologic and therefore cytoid bodies are not specific for any particular disease.\textsuperscript{57}

\textbf{Fig. 6}
Bright thick “waxy” staining of IgG in dermal blood vessels in a case of porphyria cutanea tarda. (fluorescence microscopy, original magnification x400)
IV. EPIDERMAL NUCLEAR FLUORESCENCE

In vivo anti-nuclear antibodies were observed by DIF in epithelial cell nuclei (from rat liver substrate) in patients with connective tissue diseases, most frequently mixed connective tissue disease (in 47-100%), systemic scleroderma (20%), SLE (10-15%), Sjogren’s syndrome, dermatomyositis, and rarely with rheumatoid arthritis.\textsuperscript{119, 120} (Fig. 8) Patterns of deposition may be speckled, homogeneous, nodular or combined. Immunoglobulin and/or complement may be deposited along the DEJ and in dermal blood vessels in any of these cases. This penetration of IgG into the epidermal cell nuclei has been found to be observable and not artefactual.\textsuperscript{119, 121} On the other hand, epidermal keratinocyte intracytoplasmic particulate IgG staining correlates to anti-Ro/SSA antibodies and may be found in SCLE, Sjogren’s syndrome and mixed connective tissue disease.\textsuperscript{120}

1. MIXED CONNECTIVE TISSUE DISEASE

Mixed connective tissue disease (MCTD) is diagnosed when overlapping clinical features or SLE, systemic sclerosis and dermatomyositis are found along with characteristic serologic findings of high titers of anti-U1RNP (uridine-rich ribonucleoprotein) antibodies.\textsuperscript{122} Anti-U1RNP antibodies are detected in up to 100% of patients with MCTD and are confirmatory of the diagnosis. They are usually associated with presence of sclerodactyly, Raynaud’s phenomenon, esophageal dysmotility, pulmonary dysfunction and arthritis.\textsuperscript{59} MCTD has a female ratio of about 16:1, and a mean age of onset of 29 years, and a duration of up to 6 years. Skin lesions may be variable and range from photodistributed erythematous annular or papulosquamous lesions resembling LE, diffuse non-scarring alopecia, abnormal pigmentation, sclerodactyly, palpable purpura and porphyria-like blisters.\textsuperscript{123, 124} Associated clinical findings include lymphoadenopathy and high levels of polyclonal immunoglobulins, lymphopenia and positive rheumatoid factor.\textsuperscript{123} Histopathology reflects the type of skin lesion biopsies, from a lichenoid interface dermatitis, necrotic keratinocytes, vasculopathy with luminal thrombosis, pustular vasculitis or a cell-poor subepidermal blisters.\textsuperscript{125}

DIF of in vivo epidermis from normal unexposed skin or in vitro organ-culture
studies show particulate or speckled keratinocyte nuclear staining with IgG and C5b-9 in 47-100% of MCTD cases, and are associated with high titers of circulating antibodies to ribonucleoprotein (RNP). These epidermal nuclear depositions seem to correlate to anti-ENA antibodies which are directed against histidyl-transfer RNA synthetase, and are speculated to be due to relocation of nuclear and cytoplasmic Ro/SSA antigens onto cell surface as a response to UV, viral or normal exposures.

Other DIF findings include immunoglobulin deposition at the dermoeipidermal junction, and homogeneous vascular staining. Intense granular deposition of immunoreactants along DEJ is seen in almost all cases, with granular dermal vascular deposits. Granular nuclear and cytoplasmic IgG and C5b-9 deposition in seen in keratinocytes of 100% of patients with MCTD (corresponding to antibodies to ENAs like RNP) and some patients with SLE and SCLE, possibly due to complement binding to keratinocytes, inducing pores and mediating antibody-dependent cellular immunity involved in keratinocyte injury.

2. SYSTEMIC SCLERODERMA

Systemic scleroderma (SSc) is an autoimmune disorder characterized by fibrosing of skin, blood vessels and viscera like lungs, kidney and heart. It is classified into diffuse, intermediate and limited forms. American College of Rheumatology (ACR) critiera includes major features like proximal scleroderma (thickening and tautness of finger skin), and minor features such as sclerodactyly, pitting scars of the digit and bibasilar pulmonary fibrosis. Leroy and Medsger in 2001 proposed additional criteria that could increase the diagnostic sensitivity of the ACR criteria and lead to early diagnosis of SSc, including nailfold capillaroscopic changes and serologic findings (anti-centromere, anti-topoisomerase I, anti-fibrilllin, anti-PM, anti-fibrilllin, and anti-RNA polymerase antibodies). ANA will usually be positive and correlates with the presence of epidermal nuclear staining.

Scleroderma is a prototypic antiendothelial cell antibody syndrome, where immune complex or antibody-dependent cell cytotoxicity immune reaction against endothelium is activated. These anti-endothelial cell antibodies or circulating immune complexes trigger immune-mediated vascular injury. Disease-specific antibodies usually correlate to organ involvement. Anti-Scl70 antibodies are characteristic of systemic sclerosis and help differentiate patients with extensive skin and systemic involvement from limited disease but are found in only 10-20% of Ssc patients by radial immunodiffusion. Antibodies against ScI70 are directed against topoisomerase-I enzyme, a 100 kDa basic protein and presence of anti-Scl70 antibodies are related to onset of pulmonary fibrosis; anti-centromere antibodies are characteristically found in patients with CREST syndrome or are associated with pulmonary hypertension; and RNA-polymerase antibodies are associated with increased risk of renal crisis.

About 15-68% of SSc patients will have a positive DIF, showing IgM deposition at the BMZ (positive LBT) indicative of an aggressive clinical course. Also, a prominent DIF pattern will be IgM and IgG or complement deposits within epidermis nuclei with a dense speckled pattern of staining. DIF findings were unrelated to clinical type, lesion duration or ANA positivity. In systemic sclerosis, elevated serum levels of B-cell activating factor (BAFF, a B-cell survival factor belonging to tumor necrosis factor family) is found. Serum BAFF levels as determined by ELISA were found to be elevated in scleroderma patients over healthy controls (higher in generalized over
localized forms), and correlated with anti-histone antibody levels and skin lesion severity, suggesting that this is an important mediator in scleroderma.  

CONCLUSION

DIF remains the most important first step in the diagnosis of autoimmune blistering diseases. The disorders discussed in this article are summarized in Table 1. The pattern of fluorescence can lead to a narrower list of differentials, and newer adjunct immunopathologic techniques can then lead to a more accurate diagnosis. The complete diagnostic algorithms are present in (Fig. 9, 10).

In the subepidermal blistering diseases, linear deposition of immunoreactants at the BMZ points to either the pemphigoid group, EBA, bullous LE, and LAD. It is crucial to

Table. 1 Outline.

I. BASEMENT MEMBRANE ZONE DEPOSITS
   A. BASEMENT MEMBRANE ZONE STAINING: LINEAR DEPOSITION
      1. Pemphigoid group
         a. Bullous pemphigoid (BP)
         b. Cicatricial/mucous membrane pemphigoid (MMP) and anti-epiligrin cicatricial pemphigoid (AECIP)
         c. Pemphigoid (herpes) gestationis (PG)
         d. Drug-induced pemphigoid
      2. Epidermolysis Bullosa Acquisita (EBA)
      3. Linear IgA Bullous Dermatosis (LAD)
         a. Drug-induced LAD
   B. BASEMENT MEMBRANE ZONE STAINING: GRANULAR DEPOSITION
      1. Systemic Lupus Erythematosus (SLE)
         a. Bullous systemic LE (BSLE)
         b. Drug-induced LE
      2. Dermatitis Herpetiformis (DH)
      3. Drug eruptions
      4. Erythema Multiforme (EM)
      5. False positives (rosacea, drug eruption, leprosy, sun damage, others)
   C. BASEMENT MEMBRANE ZONE STAINING: SHAGGY FIBRINOGEN
      1. Lichen planus
         a. Lichen planus pemphigoides
         b. Lichen planopilaris

II. VASCULAR STAINING
   A. VASCULAR STAINING: GRANULAR DEPOSITION
      1. Immune Complex Vasculitis (ICV)
   B. VASCULAR STAINING: HOMOGENEOUS DEPOSITION
      1. Porphyria cutanea tarda (PCT)

III. CYTOID BODIES (LP, LE, EM)

IV. EPIDERMAL NUCLEAR NUCLEAR STAINING
   1. Mixed Connective Tissue Disease (MCTD)
   2. Scleroderma
Perform salt split skin on these lesions to set apart the pemphigoid group from the other conditions. IIF and IB will likewise be helpful, however the newer commercially available ELISA to the non-collagenous (NC16a) domain of BPAG2 (180 kDa, the major target epitope in the pemphigoid group), offers a rapid, highly sensitive and specific way to confirm the diagnosis of BP, MMP, anti-epiligrin cicatricial pemphigoid, lichen planus pemphigoides, and PG. EBA will show differences in DIF, with strong linear deposition of IgG at the BMZ, on salt split skin with immunoreactants staining the floor of the induced blister, and IB identifying a 290kDa antigen. The NC1 domain of type VII collagen found in anchoring fibrils is the major target antigen in EBA and bullous LE, and ELISAs using either complete NC1 domain or its component epitopes can confirm the presence of autoantibodies in either condition.

In DH, a pathognomonic granular deposition along the BMZ will be found. An adjunctive IIF method to detect IgA anti-endomysial antibodies as well as an ELISA that detects autoantibodies to tTG has been recommended for combined use to increase diagnosis in suspected DH patients with a negative DIF.

LP presents a characteristic shaggy deposition of fibrinogen at the BMZ along with cytoid bodies. Modified IF techniques using various substrates have been used to detect suspected LP antigens, such as the “lichen planus specific antigen” in the granular layer, as well as “anti-nuclear antibodies to stratified epithelia”. ELISA to desmogleins has yielded positive results in patients with erosive oral LP.

Fig. 9
Diagnostic algorithm of linear basement membrane zone deposits on immunofluorescence.
Vascular deposition of immunoreactants, if granular point to the diagnosis of immune complex vasculitis, while homogeneous deposition points to the porphrias. ELISAs to neutrophilic granule enzymes were developed to diagnose necrotizing vasculitis like WG and CSS. An IIF technique using anti-C5b-9 has been used as well in the diagnosis of PCT.

DIF findings are being described more for other dermatologic conditions such as EM and drug eruptions. It should be emphasized that patterns such as cytoid body deposition (seen in LP, LE, EM, dermatomyositis, toxic epidermal necrolysis and so on) and epidermal nuclear fluorescence (seen in MCTD, LE and scleroderma) should be considered as important diagnostic clues as major patterns such as intercellular keratinocyte staining and BMZ staining.

While the future of diagnostic immunopathology lies in the identification of specific target antigens for the development of ELISAs to detect circulating autoantibodies, traditional techniques like DIF, IIF and IB will always play a crucial role as screening tools to lead to more specific diagnosis in many autoimmune dermatologic diseases.

REFERENCES
6. Schumann H, Aman U, Tassanen K et al.: A child with localized vulval pemphigoid and IgG autoantibodies targeting the C-terminus of col-

DERMAL BLOOD VESSEL STAINING

VASCULAR STAINING

GRANULAR

IgG, IgM, IgA, C3 (2 or more)

WAXY/HOMOGENEOUS

IgA + LINEAR, BMZ STAINING

IMMUNE COMPLEX VASCULITIS

HENOECH-SCHONLEIN PURPURA

PORPHYRIA

Fig. 10
Diagnostic algorithm of dermal blood vessel staining on immunofluorescence.
Bullous pemphigoid induced by matol to react to both the epidermal and dermal side of mucous membrane pemphigoid: the pathogenic relevance to HLA class II alleles and disease severity. Br J Dermatol 154: 90-98, 2006.


80. Finan MC, Schroeter AL: Cutaneous immunofluorescence study of erythema multiforme: