

**IMMUNOHISTOCHEMISTRY VERSUS
IMMUNOFLUORESCENCE IN THE DIAGNOSIS OF
AUTOIMMUNE BLISTERING DISEASES**Ana Maria Abreu Velez¹, Paul B. Googe, Jr.², Michael S. Howard¹¹Georgia Dermatopathology Associates, Atlanta, Georgia, USA²Knoxville Dermatopathology Laboratory, Knoxville, Tennessee, USA**Source of Support:**

Georgia Dermatopathology Associates, Atlanta, Georgia, USA (MSH, AMAV), Mineros SA, Medellin and Embassy of Japan in Colombia, Medellin (AMAV), and Knoxville Dermatopathology Laboratory, Knoxville, Tennessee, USA (PBG).

Competing Interests:
None

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Our Dermatol Online. 2013; 4(Suppl. 3): 585-595

Date of submission: 10.06.2013 / acceptance: 05.08.2013

Abstract

Introduction: In patients with autoimmune skin blistering diseases (ABDs), the diagnostic gold standard has classically been direct and indirect immunofluorescence (DIF and IIF), despite inherent technical problems of autofluorescence.

Aim: We sought to overcome autofluorescence issues and compare the reliability of immunofluorescence versus immunohistochemistry (IHC) staining in the diagnoses of these diseases.

Methods: We tested via IHC for anti-human IgG, IgM, IgA, IgD, IgE, Kappa light chains, Lambda light chains, Complement/C3c, Complement/C1q, Complement/C3d, albumin and fibrinogen in 30 patients affected by a new variant of endemic pemphigus foliaceus in El Bagre, Colombia (El Bagre-EPF), and 30 control biopsies from the endemic area. We also tested archival biopsies from patients with ABDs whose diagnoses were made clinically, histopathologically and by DIF/IIF studies from 2 independent dermatopathology laboratories in the USA. Specifically, we tested 34 patients with bullous pemphigoid (BP), 18 with pemphigus vulgaris (PV), 8 with pemphigus foliaceus (PF), 14 with dermatitis herpetiformis (DH) and 30 control skin samples from plastic esthetic surgery reduction surgeries.

Results: The diagnostic correlation between IHC and DIF-IIF was almost 98% in most cases. IHC revealed evidence of autofluorescence around dermal blood vessels, dermal eccrine glands and neurovascular packages feeding skin appendices in ABDs; this autofluorescence may represent a non-specific immune response. Strong patterns of positivity were seen also in endothelial-mesenchymal cell junction-like structures, as well as between dermal fibrohistiocytic cells. In PV, we noted strong reactivity to neurovascular packages supplying sebaceous glands, as well as apocrine glands with edematous changes.

Conclusions: We suggest that IHC is as reliable as DIF or IIF for the diagnosis of ABDs; our findings further suggest that what has previously been considered DIF/IIF autofluorescence background may be of relevance in ABDs. Our discovery of reactivity against edematous dermal apocrine glands may be related to the fact that PV has a vegetant form, with lesions present in anatomic areas where these glands exist.

Key words: autoimmune blistering skin diseases; autofluorescence; immunohistochemistry

Abbreviations and acronyms: Bullous pemphigoid (BP), immunohistochemistry (IHC), direct and indirect immunofluorescence (DIF, IIF), hematoxylin and eosin (H & E), basement membrane zone (BMZ), intercellular staining between keratinocytes (ICS), pemphigus vulgaris (PV), autoimmune blistering skin disease (ABD), fogo selvagem (FS), endemic pemphigus foliaceus in El Bagre, Colombia (El Bagre-EPF), dermatitis herpetiformis (DH).

Cite this article:

Ana Maria Abreu Velez, Paul B. Googe, Jr., Michael S. Howard: Immunohistochemistry versus immunofluorescence in the diagnosis of autoimmune blistering diseases. *Our Dermatol Online*. 2013; 4(Suppl.3): 585-595.

Practical learning:

· IHC may be as reliable as DIF or IIF for the diagnosis of ABDs. Running positive and negative controls is recommended, utilizing paraffin blocks of similar ages to the patient cases.

· IHC reveals that ABDs may present more antigenic molecules than are classically recognized.
· IHC cannot replace antibody titers, or salt split skin techniques in combination with IIF.

Introduction

The techniques of direct and indirect immunofluorescence (DIF and IIF) are of proven value in confirming the presence of immunoglobulins, complement, and fibrinogen; in turn, these findings contribute to the diagnosis of multiple autoimmune skin diseases [1-4]. In classic ABD immunofluorescence testing, a single fluorophore (fluorescein isothiocyanate; FITC) has been utilized, and it has been accepted that background autofluorescence exists [5]. Further, it is assumed that diagnostic DIF/IIF reactivity in ABD patients will vary depending on concomitant administration of therapeutic immunosuppressor agents [6].

In addition, correlation of serum antibodies with disease severity in pemphigus and bullous pemphigoid (BP) via IIF is widely utilized [8,9]. Because correlating paraffin block biopsies are available in most dermatological services, we attempted to compare the diagnostic results obtained from DIF, IIF and immunohistochemistry (IHC) in several ABDs.

Materials and Methods

Subjects of study

We tested 30 biopsies from patients affected by endemic pemphigus foliaceus in El Bagre, Colombia (El Bagre-EPF); diagnostic criteria were followed as previously described [8-10]. We also tested skin biopsies from 30 controls from the El Bagre EPF endemic area, and 30 additional control skin samples from cosmetic surgery patients in the USA, taken from the chest and/or abdomen. Biopsies were initially fixed in 10% buffered formalin, then embedded in paraffin and cut at 4 micron thicknesses. The tissue was then submitted for hematoxylin and eosin (H&E) and IHC staining. We also tested ABD cases from archival files of two private, board certified dermatopathology laboratories in the USA. Our patients were diagnosed clinically by the referring physicians, by H&E staining, and by DIF and IIF. We did not record the age of the biopsies, nor if the patients were taking immunosuppressive therapeutic medications at the time of the biopsy. We evaluated 34 biopsies from bullous pemphigoid (BP) patients, 4 from patients with pemphigus vulgaris (PV), 8 from patients with sporadic pemphigus foliaceus (PF), and 14 from patients with dermatitis herpetiformis (DH). For all of the El Bagre area patients and controls we obtained written consents, as well as Institutional Review Board permission. The archival biopsies were IRB exempt due to the lack of patient identifiers.

Quantification of staining intensity to obtain precise data on IHC parameters

We utilized the following algorithm: area of positive signal divided by the area studied. The staining intensity of these antibodies was also evaluated qualitatively by two independent observers. We utilized the following traditional categories to classify reactivity: intercellular staining between keratinocytes (ICS) and basement membrane staining (BMZ). In addition to these patterns, we added the following: upper dermal blood vessel perivascular staining (UVS), neurovascular staining around skin appendageal structures (NVS), endothelial-mesenchymal cell junction-like staining (EMCJ), dermal cell junction staining (DCS) and peritelocyte staining (ATS) [11]. Our IHC staining

was performed as previously described before [7-10]. For IHC, all antibodies utilized were obtained from Dako (Carpinteria, California, USA). A summary of the antibodies utilized, their dilutions, catalogue numbers and methods of antigen retrieval show in Supplementary Table I.

Statistical methods

Differences in staining intensity and positivity were tested using a GraphPad Software statistical analysis system, and employing Student's t-test. We considered a statistical significance to be present with p values of 0.05 or less, assuming a normal distribution of the samples.

Result

In most of the ABDs, our results followed established DIF and IIF patterns with statistical significance in comparison to controls run with similar markers (p values of less than 0.05). Our summarized results shown in Table I. In addition to the classical patterns of reactivity appreciated by DIF and IIF, multiple additional patterns of positivity were seen. The most common one featured positive staining around the upper dermal blood vessels in the ABD patients, again with statistical significance in comparison to controls (p = 0.0001). IgG, IgM, Complement/C3c, Complement/C1q and fibrinogen were the most common positive markers detected in this pattern. Most of the ABDs were positive in this pattern for more than 3 markers at a time. In BP, we also observed strong perivascular positivity in the intermediate and deep dermis with similar markers as those described above (Fig. 1-4). In BP, PV, El Bagre-EPF and PF, we noted strong positivity to neurovascular structures feeding skin appendageal structures, especially in eccrine glands and pilosebaceous units. In PV, this phenomenon was also observed in neurovascular packages supplying and surrounding dermal apocrine glands; on H&E review, these glands were also noted to be edematous with acantholysis-like features (p<0.05) (Fig. 1-4). We classified our findings as negative (-), weakly positive (+), positive (+++) and strongly positive (++++).

Positivity was also observed in most ABDs in what seemed to be cell junction-like structures between endothelial cells in the dermis and the surrounding mesenchymal extracellular matrix (p<0.05) (Fig. 1-4). The degree of positivity varied depending of the size of the vessels and their relative deepness in the dermis (Tabl. I). In BP, such those that seems to be in the junctions between the dermal cells junctions with stronger positivity in the middle of the dermis. The normal controls did not show this staining, with exception of deposits of IgG, IgM and albumin in the dermis; however, this staining was weaker and without any specific pattern. In several active clinical cases of El Bagre-EPF, we also noted positive staining to some kind of cell junction-like structures in piloerector muscles with both IgG and IgM. Notably, the reactivity against the apocrine glands and the H&E alterations of edema and acantholytic-like changes may be related with the fact that PV has a vegetant clinical form, with lesions anatomically present in areas where these glands predominate.

In Table I and Figures 1 through 4, we summarize our primary results.

Antibody	BP n=34	PV n=14	PF n=4	DH n=10	EI Bagre-EPF n=30	Controls from Endemic area n=30	Skin plastic Surgery controls n=15
IgA	Positive around some dermal blood vessels. Some epidermal keratinocytes showed some cytoplasmic staining in several biopsies (+).	Some positive cells debris inside the blister. Some positive blood vessels in the upper dermis and some individual cells in the upper dermis. Also positive on blood vessels around the eccrine glands and on vessels of the septae of subcutaneous adipose tissue.	Some small dermal blood vessels positive. Also, positive on some small blood vessels around eccrine sweat glands (+).	Positive below subepidermal blisters, mainly in the papillary dermis (+++). Positive in some linear areas in the epidermal stratum corneum (++). Also positive in some areas of the intracellular matrix bundles (++). Some positivity in neurovascular packages feeding sebaceous glands (++).	Some patients with positive staining around upper and intermediate dermal blood vessels (++).	Negative.	Negative.
IgG	BMZ linear positivity on roofs and floors of the blisters, with more positivity on the roofs (34/34). Some areas of the epidermis showed pericytoplasmic staining in keratinocytes (22/34). Sometimes positive perinuclear staining as well. Positive staining also noted around some small dermal blood vessels. Some areas of the papillary dermis extracellular matrix and upper dermis showed reactivity (+++). Several fibroblastoid cells positive through the entire dermis. Many deep nerves positive in the epineuria.	ICS, some upper and around several neurovascular vessels. Positive around some vessels around the sweat glands upper dermis and small vessels in the septae of the fatty tissue (++). Positive some extracellular matrix fibers especially in the upper and intermediate dermis. Positive also some small vessels around the sweat glands area (+).	ICS between epidermal keratinocytes, mostly in upper layers. Positive staining of some small upper dermal blood vessels. Positive staining around some dermal blood vessels and eccrine glands (++).	Similar distribution as IgA; also in several vessels.	Positive in several cases in epidermal stratum corneum (++). Also, some intracytoplasmic positive staining within epidermal keratinocytes. Positive ICS, mainly in epidermal stratum granulosum in acute and relapsing cases. Positive in several cases on upper dermal blood vessels (++). Chronic cases demonstrated some positive staining against mesenchymal-endothelial cell junction-like structures in the dermis, as well as around some dermal eccrine glands.	Negative.	Negative.
IgM	Some areas of ICS-like staining and some areas of pericytoplasmic staining in epidermal keratinocytes (21/34). Also, positive staining around upper blood vessels and the dermal extracellular matrix (+++). The pattern of this immunoglobulin is very similar to that seen with IgG.	Some epidermal subcorneal reactivity in several areas. Focal epidermal ICS staining is noted in several spots. Positive staining also noted around several dermal blood vessels in the dermis, around some connective tissue and some deep neurovascular tissue around eccrine sweat glands, sebaceous glands and adipose septae (+++).	Positive ICS between epidermal keratinocytes, mostly in upper layers and positive around some small upper dermal blood vessels. Also positive around some blood vessels around eccrine glands (++). Positive around sebaceous and eccrine gland neurovascular supplies.	Similar distribution as with IgA. In some biopsies, some intercellular keratinocyte staining was observed. Also, some reinforcement was noted around hair follicles.	Some cases displayed spotty positive staining in the epidermal corneal layer. Some epidermal keratinocytic ICS in several cases (++). Some epidermal keratinocyte spotty pericytoplasmic positive staining in several areas of the epidermis, and some BMZ staining. In most chronic cases, positive staining in a band-like distribution in the upper dermis and/or intermediate dermis, including on blood vessels (++). Reinforcement of the mesenchymal-endothelial cell junction-like structures and cells and telocyte-like structures also seen.	Negative.	Negative.

Table I. Cell populations and markers in lesional skin from multiple autoimmune skin diseases.

Antibody	BP n=34	PV n=14	PF n=4	DH n=10	EL Bagre-EPF n=30	Controls from endemic area n=30	Skin plastic Surgery controls n=15
IgE	Linear on both floors and roofs of blisters in many cases (23/34). Positive (+++) intracytoplasmic, perinuclear staining in some epidermal keratinocytes.(26/34). Positive staining on some individual cells in dermis, and focally around upper dermal blood vessels (++)	Positive nuclear and focal cytoplasmic staining in epidermal keratinocytes. Also, positive on several large cells in the upper dermal inflammatory infiltrate. Positive staining of some vessels around eccrine glands. (++)	Positive nuclear and focal cytoplasmic staining in epidermal keratinocytes Also positive staining on several large cells in the upper dermal inflammatory infiltrate. Positive staining of some vessels around the eccrine glands (++)	Most cases were negative. Some positive staining in the upper dermal inflammatory infiltrate. Two cases were positive in sebaceous glands, in plasmacytoid cells in the dermis and around eccrine glands.	Several cases, both acute and chronic, displayed positive staining on individual cells, mainly around the upper dermal blood vessels (++) . Mesenchymal-endothelial cell junction-like structures and telocyte-like structure positive staining was also noted, as well as around some dermal eccrine glands.	Negative.	Negative.
IgD	Positive linear staining in several biopsies along the BMZ, mostly on blister floors. Positive on several fibroblastoid cells in the dermis. Positive around several dermal blood vessels; superficial, intermediate and deep (++++) (20/34).	Some positive staining on individual large cells in the upper dermal perivascular inflammatory infiltrate, and on some of the upper dermal blood vessels (+). Some epidermal ICS positivity and some positive staining on cells inside the blisters.	Positive in several upper dermal small blood vessels, and on some blood vessels around eccrine glands. Some scattered staining around very actively inflammatory epidermal blisters (+).	Several cases followed the same pattern as IgA, including the positivity in the dermal papillae, and blood vessels. Two cases were positive in the sebaceous glands, in plasmacytoid cells in the dermis and around the eccrine glands.	Positive staining pattern followed the distribution of the stronger immunoglobulins, including some epidermal keratinocytic intracytoplasmic positive staining, and some staining on upper dermal blood vessels. In some cases, some spotty positivity along the BMZ and some staining around selected eccrine gland ducts.	Negative.	Negative.
Complement/C3c	Positive linear deposits at blister splits, primarily on blister roofs but also some on blister floors (++) in 34/34 cases. Also around several small and large dermal neurovascular packages (+++). Positive staining also present around eccrine ducts and BMZ of eccrine ducts, as well as on blood vessels around the hair follicles (++) . Some reactivity also seen in the upper epidermal corneal layer (+).	Epidermal ICS, and staining on some upper dermal blood vessels and several dermal neurovascular packages. Some positivity in focal areas of the epidermal corneal layer. Also positive staining on some fibroblastoid cells in the dermis. Positive focal staining on BMZs of the sebaceous glands and on their neurovascular supply packages. Positive staining on some small blood vessels in the deep connective tissue (++)	Positive staining on several blood vessels in the upper and intermediate dermal plexus. Positive in some small blood vessels in the deep connective tissue. Positive around neurovascular packages of dermal sebaceous and eccrine glands (++)	Positive deposits in the upper and lower dermal tissue (+++). Positivity also noted in the epidermal corneal layer. Some epidermal keratinocyte ICS and/or cytoplasmic staining. Staining in the extracellular matrix and around eccrine glands and ducts. Multiple fibroblastoid cells were positive in the dermis.	Some spotty positive staining on the epidermal corneal layer. Some epidermal positive ICS in several cases (++) . BMZ staining also in several cases (++) . Positive staining in upper dermis and on neurovascular packages of all skin appendageal structures (++) . Positive staining in multiple cases on mesenchymal-endothelial cell junction-like structures, and on telocyte-like structures.	Negative.	Negative.
Complement/C3d	Positive around several small and large dermal blood vessels. Positive linear BMZ staining on blister floors and roofs (+++)	Positive around several upper dermal blood vessels. Some epidermal ICS, positive in focal areas (++) . Positive staining inside the blisters. Positive staining around neurovascular supplies of pilosebaceous glands units. Some extracellular matrix staining, positive in the intermediate dermis.	Positive around several upper dermal blood vessels. Some epidermal ICS, positive in few areas (++) . Positive staining around dermal sebaceous and eccrine gland neurovascular supplies.	In some biopsies, staining followed the pattern of positivity of IgA, although with weaker intensity.	Positive in the majority of the cases in most vessels in dermis.	Negative.	Negative.

Table I. Cell populations and markers in lesional skin from multiple autoimmune skin diseases (continued).

Antibody	BP n=34	PV n=14	PF n=4	DH n=10	El Bagre-EPF n=30	Controls from Endemic area= 30	Skin plastic Surgery controls n=15
Complement/C1q	Positive linear BMZ staining on both sides of the blisters, but primarily on blister roofs. Positive staining on focal extracellular matrix fibers, in bundles in the upper, intermediate and deep dermis (++). Also positive dermal staining on the upper neurovascular plexus (++). Some focal areas of the epidermis with cytoplasmic staining of the keratinocytes.	Positive staining on blister floors, and some inside blisters. Some staining in the upper dermal extracellular matrix. Positive staining on dermal blood vessels, sebaceous glands neurovascular supply packages (++). Several BMZs of hair follicles and sebaceous glands were also patchy positive. Positive staining around dermal eccrine glands and ducts.	Positive staining around pilosebaceous units (++). Positive staining around several upper dermal blood vessels. Some focal epidermal ICS positive staining (++). Positive staining around dermal sebaceous and eccrine gland neurovascular supplies.	Positive staining at the subepidermal blister floor, and in the papillary dermal areas (+++). Some focal epidermal dermal extracellular matrix was very reactive, accentuated in bundled groups (+++). Positive staining around some dermal neurovascular packages. In summary, the staining pattern followed a similar pattern to that of IgA.	Positive several extracellular matrix fibers, mostly intermediate ones. Positive cells-junction like of the piloerector muscle. Positive around sweat glands vessels.	Negative.	Negative.
Kappa light chains	Positive staining on blister floors and roofs, but more prominent on the floors (++). Positive staining in most areas of the dermis (++).	Positive epidermal ICS, on upper dermal blood vessels and around eccrine ducts (++).	Positive staining in the epidermal corneal layer. Positive epidermal ICS, and positive staining on upper dermal blood vessels and around dermal eccrine ducts (++). Positive staining around the sebaceous and sweat gland neurovascular supplies.	Similar distribution as IgA.	Follows same pattern than IgG and IgM combined.	Negative.	Negative.
Lambda light chains	Positive staining on blister floors and roofs, but more prominent on the floors (++). Positive staining in most areas of the dermis (++).	Positive epidermal ICS, on upper dermal blood vessels and around eccrine ducts. (++).	Positive in the epidermal corneal layer. Positive epidermal ICS, and positive staining on upper dermal blood vessels and around dermal eccrine ducts (++). Positive staining around the sebaceous and sweat gland neurovascular supplies.	Similar distribution as IgA.	Follows same pattern than IgG and IgM combined.	Negative.	Negative.

Table I. Cell populations and markers in lesional skin from multiple autoimmune skin diseases (continued).

Antibody	BP n=34	PV n=14	PF n=4	DH n=10	EL Bagre-EPF n=30	Controls from Endemic area n=30	Skin plastic Surgery controls n=15
Fibrinogen	Positive staining in the epidermal corneal layer, ICS in epidermal stratum spinosum, on upper and intermediate dermal blood vessels and around dermal eccrine glands Positive, strong band-like staining throughout the papillary dermis (+++). Also, some positivity in the deep dermal extracellular matrix. Positive staining around small dermal blood vessels, including those associated with eccrine glands. Essentially, very similar to IgG.	Positive epidermal ICS in stratum spinosum. Some positive staining inside disease blisters. Positive staining on several small blood vessels in the dermis, and around some dermal connective tissues. Positive staining in the epidermal subcorneal area. Positive staining on some deep dermal large nerves around the eccrine glands Positive strong band-like staining in the dermal extracellular matrix in several areas, and on several dermal neurovascular plexus structures (++) and deep dermal, small blood vessels. Positive staining around dermal eccrine glands. Positive staining also noted around some subcutaneous adipose tissue septae. Positive staining on the BMZs in some areas of the sebaceous glands.	Positive staining in the epidermal corneal layer, epidermal ICS, on upper and intermediate dermal blood vessels and around dermal eccrine glands Positive strong band-like staining throughout the papillary dermis (+++). Positive staining around dermal sebaceous and eccrine gland neurovascular supplies.	Similar positive staining distribution as IgA. Positive staining on neurovascular supply structures of dermal sebaceous glands (++)	Some spotty positive staining on the epidermal corneal layer in several cases (+). Some BMZ staining. Positive staining on upper dermal blood vessels (++) .Positive staining on dermal mesenchymal-endothelial cell junction-like structures and telocyte-like structures. Positive staining on cell-junction like structures in dermal piloreceptor muscles. Positive staining around dermal blood vessels supplying dermal eccrine glands	Negative.	Negative.
Albumin	Positive, strong band-like staining noted in the papillary dermis and around dermal vessels and eccrine glands (++++). Also, positive staining on the deep dermal extracellular matrix.	Positive staining on the epidermal corneal layer. Positive epidermal ICS, and upper and intermediate dermal blood vessel staining and around dermal eccrine glands and one perieccrine large nerve. Positive, strong band-like staining throughout the papillary and intermediate dermis, suggesting a compartmentalization of the overall immune response (++++).	Positive staining on the epidermal corneal layer. Positive epidermal ICS, and positive staining on upper and intermediate dermal blood vessels and around dermal eccrine glands Positive, strong band-like staining throughout the papillary dermis (++++). Positive staining around dermal sebaceous and sweat gland neurovascular supplies.	Positive strong band-like staining in the papillary dermis (++++). Positive staining in some areas of the epidermal corneal layer, and on the dermal extracellular matrix (++++). Some trace staining in the epidermis between keratinocytes, and some keratinocytic cytoplasmic staining. Followed a similar pattern as IgA, but with stronger positivity.	Followed the same pattern as fibrinogen	Non-specific staining was present in the dermis.	Negative.

Table I. Cell populations and markers in lesional skin from multiple autoimmune skin diseases (continued).

Cat No.	Antibodies (all from Dako)	Working dilution	Retrieval method (High, Low or Proteinase K(PK))
AR0423	Polyclonal rabbit anti-human IgG	Flex ready to use	High
IR153	Polyclonal rabbit anti-human IgM	Flex ready to use	High
IR510	Polyclonal rabbit anti-human IgA.	Flex ready to use	High
IR517	Polyclonal rabbit anti-human IgD	Flex ready to use	Low
A0094	Polyclonal rabbit anti-human IgE	1:800	High
A0062	Polyclonal rabbit anti-human Complement/C3c	1:1000	None
A0063	Polyclonal rabbit anti-human Complement/C3d	1:400.	PK
A0136	Polyclonal rabbit Complement /C1q	1:100.	PK
A0001	Polyclonal rabbit anti-human albumin.	1:13,000	None
A0080	Polyclonal rabbit anti-human fibrinogen	1:1000	PK
IR506	Polyclonal rabbit anti-human kappa light chains	Flex ready to use	High
IR507	Polyclonal rabbit anti-human lambda light chains	Flex ready to use	High

Table I - Supplemental. Antibodies utilized, with their respective working parameters.

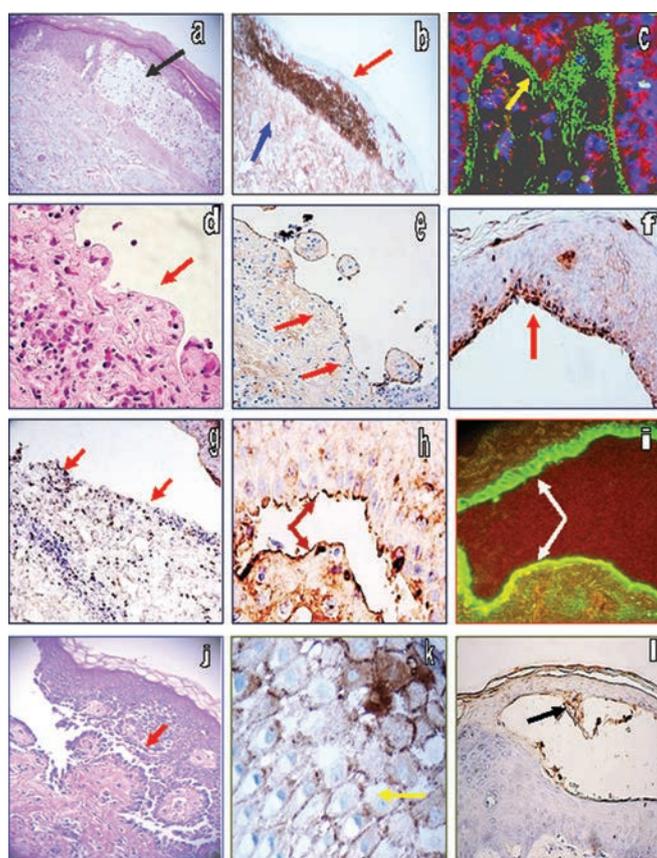


Figure 1. a. Classic H&E staining of a DH case, demonstrating a subepidermal blister (black arrow). b. IHC, demonstrating positive staining with anti-human IgA antibodies in the blister, (brown staining; red arrow), and weaker, punctate staining in the upper dermis (brown staining; blue arrow). c. DIF of the same patient as in a and b, utilizing FITC conjugated anti-human IgA and showing positive "snow on the mountains" staining in the blister and around upper dermal blood vessels (green staining; yellow arrow); epidermal keratinocyte nuclei were counterstained with Dapi (light blue). d. Classic H&E image of a BP case; note the eosinophils within the upper dermis, subjacent to a disease blister (red arrow). e. Case of BP, with IHC positive linear staining for Complement/C3 at the BMZ (dark staining; red arrows). f. Same BP case as in e, with positive linear IHC staining for IgM at the BMZ. g. BP case, with positive punctate IHC staining for IgE below a disease blister in the dermis (brown staining; red arrow). h. BP case, with positive linear IHC staining for IgG on both sides of a disease blister (brown staining; red arrows). i. The same BP case as in h, highlighting DIF positive staining of FITC conjugated IgG on both sides of a disease blister (green-yellow staining; white arrows). j. A classic case of PV, with classic H&E "tombstone" acantholytic keratinocytes along the epidermal basaloid layer (red arrow). k. A case of PV, demonstrating a positive IHC "chicken wire" pattern of ICS with IgG between epidermal keratinocytes (brown staining; yellow arrow). l. Positive IHC staining for IgG in an epidermal blister in a case of PF (brown staining; black arrow). Please also note the focal epidermal corneal staining.

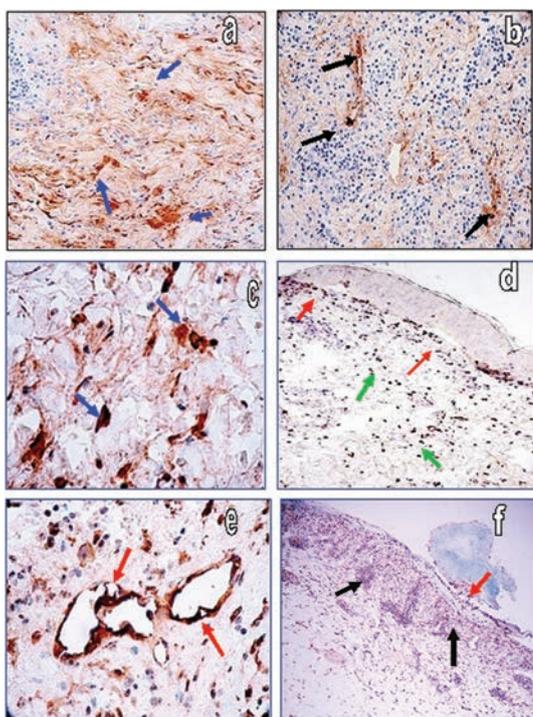


Figure 2. Atypical IHC staining patterns seen in ABDs. **a.** Complement/C3c positive IHC staining in the central dermis in a patient with BP, in ATS and/or EMCJ patterns (black arrows). **b.** Positive IHC staining for Complement/C3d in a BP patient, highlighting positive staining in several upper and intermediate dermal blood vessels (brown staining; black arrows). **c.** Many BP cases also demonstrated positive IHC IgD staining, in both ATS and EMCJ patterns (brown staining; blue arrows). **d.** Many BP cases displayed positive, punctate IHC staining for IgD along the BMZ (brown staining; red arrows), but also in the dermis in an EMCJ pattern (brown staining; green arrows). **e.** BP case, displaying positive IHC staining in a mesenchymal-endothelial junction pattern (MES) with anti-human IgD (brown staining; red arrows). **f.** In a BP patient biopsy, we utilized IHC vimentin staining to highlight compartmentalization of the inflammatory process. Note the positive linear staining at the BMZ in the floor of a disease blister (brown staining; red arrow), as well as the inflammation and structural reorganization of dermal blood vessels (brown staining; black arrows).

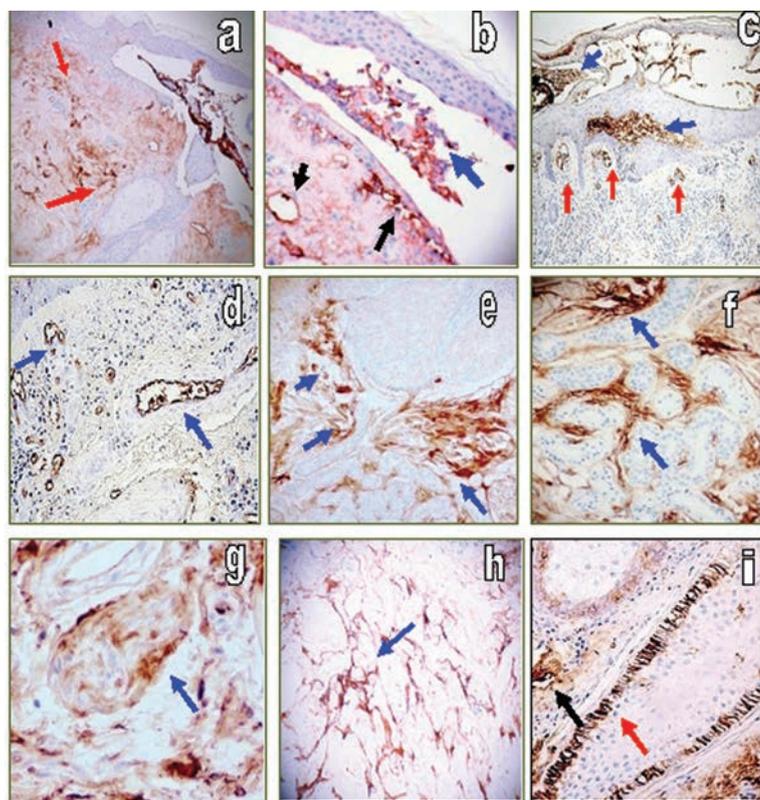


Figure 3. **a.** A PV case, demonstrating positive IHC staining for fibrinogen (note the strong dark brown staining inside an epidermal blister). In addition, note the positive staining around upper dermal inflamed blood vessels, and some parts of the extracellular matrix; this staining pattern is commonly seen in immunofluorescence and has been traditionally interpreted as autofluorescence of the vessels and dermal matrix fibers. Here, IHC is not a fluorescent method, and the observation of the identical pattern raises the possibility that the DIF staining may be due to real antigenicity not previously characterized. **b.** Same case of PV, highlighting fibrinogen reactivity in the blister (brown staining; blue arrow); also note the positive staining in the upper dermal neurovascular plexus (brown staining; black arrows). **c.** In a PF case, we noted positive IHC staining for fibrinogen in both subepidermal and subcorneal blister areas (brown staining; blue arrows), and additional staining around upper dermal blood vessels (brown staining; red arrows). **d.** In most ABDs, strong positive IHC staining was noted to small and intermediate sized dermal blood vessels, in this case with IgG (brown staining; blue arrow). **e.** Positive IHC staining for IgG around the neurovascular supply package of a sebaceous gland in a PV case (brown staining; blue arrows). **f.** Most ABDs also stained positive via IHC for Complement/C3, Complement/C3d, fibrinogen and sometimes IgG around dermal eccrine glands and their ducts; an example of a positive stain is illustrated (brown staining; blue arrows). **g.** Also, in most ABDs the neurovascular packages supplying skin appendageal structures showed positive staining with the markers documented in f. Sometimes, deep nerves also stained positive, as in this case of PV with fibrinogen (brown staining; blue arrow). **h.** Positive IHC staining with IgG in a BP patient, directed against either telocytes and/or the EMCJ (brown staining; blue arrow). **i.** A PV case, staining positive via IHC in a hair follicle for Complement/C3c (brown staining; red arrow). Note also some adjacent blood vessels with positive staining (brown staining; black arrow).

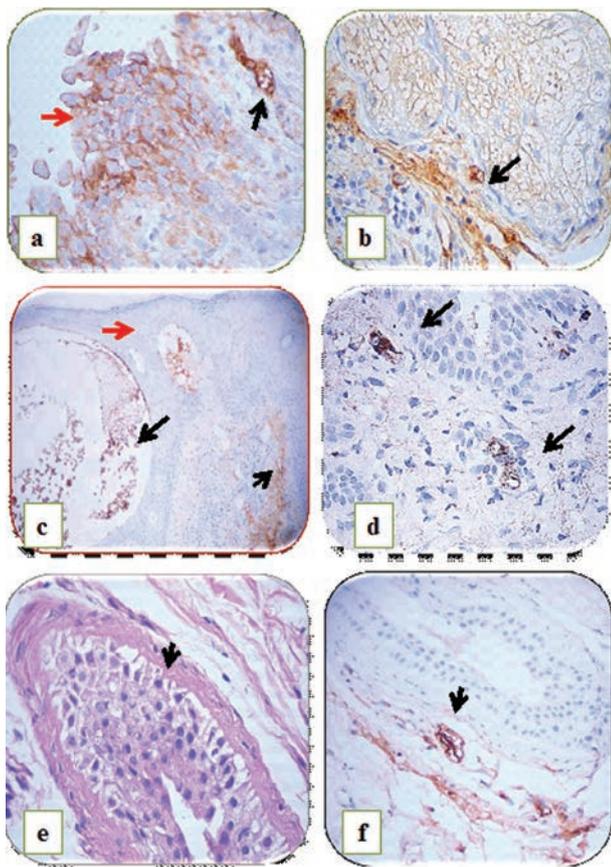


Figure 4. a. A PV case, demonstrating positive IHC staining with an ICS pattern and anti-Complement/C3c antibody between keratinocytes (brown staining; red arrow) as well as around upper dermal blood vessels (brown staining; black arrow)(400X). b. Same case as in a, demonstrating positive staining with Compliment/ C3c to neurovascular packages feeding a sebaceous gland (brown staining; black arrow) (400X). c. A BP case, demonstrating positive staining with linear deposits of complement/C3c around a subepidermal blister (brown staining; black arrow) and also in the upper dermis (brown staining; black arrow) (200X). d. A PV case, demonstrating positive staining with IgA against small vessels in the upper dermis brown stain (brown staining; black arrows)(400x). e. A PV case H&E, demonstrating edematous and acantholytic-like changes in apocrine gland cells (400X)(black arrow). f. A PV case, demonstrating positive IHC staining of a neurovascular package around an apocrine glands using anti-human fibrinogen (400X).

Discussion

DIF and IIF have been classically used for the diagnostic of ABDs; the salt split skin IIF technique was also developed to help to differentiate ABDs. IIF has been the gold standard to determine autoantibody titers that correlate with disease severity, further validated by ELISA testing [1-5]. DIF and IIF of the skin detect several autofluorescence molecules, including molecules in the extracellular matrix, blood vessels, and pigments like lipofuscin, melanin, collagen, indolamine, tryptophan, tyrosine, pyridoxine, folic acid, retinol, collagen, cholecalciferol, riboflavin and NAD(P)H [12]. The majority of these molecules fluoresce in the same UV range as FITC. In contradistinction, IHC techniques often recognize well established internal positive control staining patterns on recognized anatomic structures.

In our study, we were able to see an “edge effect” in DIF and IIF of non-specific reinforced staining around the edges of the biopsies. We were also able to determine that all the skin biopsies should be run with a control biopsy of similar age as the biopsy to be tested, especially when using older archival biopsies.

Besides the classic staining patterns seen in DIF and IIF and detected by IHC with similar specificity and sensitivity (such ICS and/or BMZ staining), we also observed other patterns that we describe as non-classic patterns. These patterns include positivity to the vessels of the dermal neurovascular packages feeding skin appendageal structures, and positivity to junctions between endothelial cells and the surrounding mesenchymal tissue. Other patterns included positivity to cell junctions within the dermis, and a pattern that we described as a telocyte-like pattern [11]. We didn't find these patterns in the controls, with

the exception of some even, nonspecific staining of IgG and IgM within the dermis.

Other authors have reported studies utilizing IHCs in ABD diagnosis, with similar results to ours in the classical patterns [14-17]. Our non-classic patterns of positivity have been also documented in DIF and IIF, especially when using multicolor and confocal microscopy (not routinely utilized in most immunodermatology laboratories). In theory and based on other studies, these autofluorescence molecules should not be detected by IHC [10,17-21]. As noted, besides the classical patterns of reactivity we noticed additional patterns such as UVS. The increased reactivity of dermal blood vessels and/or molecules involved in the transit of inflammatory markers through the dermal blood vessels has been previously shown to possibly play roles in ABDs [19-21].

The reactivity of patients with ABDs to dermal blood vessels and/or endothelial cells with strong inflammatory and immune activation markers, including autoantigens to plakophilins 3 and 4 (present in dermal blood vessels) has been also previously reported [23-29].

Some authors have shown DH to have evidence of endothelial cell activation in the skin, and systemic manifestations of the ongoing inflammation associated with the mucosal immune response. Endothelial cell activation may play a critical role in the development of skin lesions in patients with DH [27].

Other authors investigated the expression of vascular permeability factor (VPF), that plays an important role in increased vascular permeability and angiogenesis in three bullous diseases; these diseases feature subepidermal blister formation characterized by hyperpermeable dermal microvessels and pronounced papillary dermal edema [28].

The expression of VPF mRNA was strongly up-regulated in the lesional epidermis of BP (n = 3), erythema multiforme (n = 3), and DH (n = 4) as detected by in situ hybridization studies. Epidermal labeling was particularly pronounced over blisters, but strong expression was also noted in areas of the epidermis adjacent to dermal inflammatory infiltrates distant from the blisters. Moreover, the VPF receptors were upregulated in endothelial cells in superficial dermal microvessels. High levels of VPF were detected in blister fluids obtained from patients with BP. The findings described by these authors strongly suggest that VPF plays an important role in the induction of increased microvascular permeability in bullous diseases; leading to papillary edema, fibrin deposition and blister formation in these disorders [28]. Therefore, the positive staining to dermal blood vessels and neurovascular supply packages may be detecting secondary antigens.

Previous authors also reported that soluble E-selectin (sE-selectin), an isoform of the cell membrane protein E-selectin (an adhesion molecule synthesized only by endothelial cells) is significantly increased in sera of patients with BP and PV [29]. Other authors recently investigated endothelial cell activation via gene profiling in patients affected by DH (e.g. SELL, SELE genes) that code for cell surface components, specifically members of a family of adhesion/homing receptors that play important roles in lymphocyte-endothelial cell interactions. The gene activation was increased, as well as neutrophil extravasation. The SELL/SELE coded molecules are composed of multiple domains: one homologous to lectins, one to epidermal growth factor, and two to the consensus repeat units found in Complement C3/C4-binding proteins [30]. Our findings of positive reactivity in dermal blood vessels, especially in the upper dermis and neurovascular skin appendices are suggestive of possible antigenicity for these structures in ABDs. Finally, recent studies have shown positive reactivity to eccrine and sebaceous glands in ABDs utilizing IIF and DIF [31-35].

In regard to the positive patterns seen in the dermis (possibly associated with cell junctions and/or telocytes), we were able to recently demonstrate activation of both multiple proteases and protease inhibitors following the same patterns of positivity we are describing here [36]. Electron microscopy studies using antibodies and colocalization will help to see if these non-classical patterns of reactivity seen in ABDs are artifacts, or possibly associated with pathological effects in these diseases.

Acknowledgements

Jonathan S. Jones, HT (ASCP) at Georgia Dermatopathology Associates for excellent technical assistance.

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