Direct immunofluorescence microscopy

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Direct Immunofluorescence Microscopy

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Learning Objectives

After studying this chapter, you should know:

- The various cutaneous immunodeposition patterns in pemphigus, pemphigoid, dermatitis herpetiformis and porphyria
- The difference between an n-serrated and u-serrated pattern in pemphigoids

Introduction & Aims

Ever since the discovery of the presence of autoantibodies in pemphigus in 1964 by Beutner and Jordon [1], immunofluorescence microscopy has become an essential part in the diagnostics of blistering diseases. Both serum and biopsy specimens can be examined by this method. The next chapter will describe the technique of direct immunofluorescence microscopy, i.e. visualization of *in vivo* bound autoantibodies. After reading this chapter the reader knows the different patterns that can be recognized in various blistering diseases.

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Laboratory Preparation

The purpose of direct immunofluorescence microscopy is to detect *in vivo* antibodies. This is done by adding a fluorescent labeled antibody against a human antigen, e.g. a goat antibody directed against human IgG, on a frozen section. To prepare a skin or mucosa biopsy for immunofluorescence microscopy the following steps are recommended (Groningen protocol):

- Cut frozen sections at a thickness of 4 um.
- Blow dry the sections with a cold dryer for 15 min
- Rinse the slides with PBS (NaCl 8.75 g/l, Na2HPO4 1.14 g/l, KH2PO4 0.27 g/l) for a minimum of 5 seconds. Wipe off excess PBS.
- Place fluorescent isothiocyanate (FITC)-conjugated antibody on the slides and incubate in a moist chamber for 30–40 min. See Table 4.1 for used antibodies.
- Rinse the slides with PBS and wash the slides subsequently for 30 min in PBS.
- Wipe off excess PBS.
- Place bisbenzimide (Hoechst 33258), which binds to double stranded DNA and therefore provides a nuclear staining, on the slides and incubate for 5–10 min on room temperature.
- Rinse the slides with PBS and wash the slides subsequently for 30 min in PBS.
- Place a drop of PBS/glycerin (1:1) on each section and top with a cover slip.

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Table 4.1 Recommended FITC conjugated antibodies

Antibodies	Manufacturer
FITC-conjugated Goat F(ab)2 anti-human IgG	Protos 311, Protos immunoresearch, Burlingame, CA, US
FITC-conjugated Goat F(ab)2 anti-human IgA	Protos 312, Protos immunoresearch, Burlingame, CA, US
FITC-conjugated Goat F(ab)2 anti- human IgM	Protos 313, Protos immunoresearch, Burlingame, CA, US
FITC-conjugated Rabbit anti-human fibrinogen	Dako F111, Dako, Glostrup, Denmark
FITC-conjugated Rabbit anti-human C3c complement	Dako F201, Dako, Glostrup, Denmark

Immunofluorescence Patterns

Pemphigus

Pemphigus is caused by autoantibodies directed against desmosomal antigens, in particular desmoglein 1 (pemphigus foliaceus) or desmoglein 3 (mucosal pemphigus vulgaris) or desmoglein 1 and 3 (mucocutaneous pemphigus vulgaris) [2], although cases have been described with antibodies against desmocollin 1 or 3. Whatever the nature of the antibodies or the pemphigus variant, direct immunofluorescence of pemphigus shows depositions of immunoglobulins and/or complement on the epithelial cell surface (ECS) in virtually all patients [3]. This ECS deposition is in most cases throughout the entire epidermis and mucosal epithelium, therefore a subclassification can not be made. In the majority of textbooks this is described as a smooth pattern throughout the epidermis (Fig. 4.1a). However, in many biopsies a fine or coarse granular pattern can be observed (Fig. 4.1b, c). *Pemphigus is characterized by ECS deposition of immunoglobulins and complement in a smooth or granular pattern* These clusters seem to be the result of clustering of IgG, Dsg3 and plakoglobin, but no other desmosomal components are involved [4]. Due to its bivalency, IgG crosslinks non-junctional Dsg molecules and these crosslinked molecules then concentrate in dots. In addition to deposits throughout the epidermis, immunoglobulins can in many cases also be observed in adnexal structures, e.g. hair follicles and sweat glands. False positive ECS deposition can be observed in biopsies of eczema lesions. In these cases a "tram rails" pattern between the keratinocytes can be observed in contrast to the smooth or granular patterns in pemphigus (Fig. 4.2).

On top of ECS deposition, in some cases also a granular deposition of autoantibodies and complement can be found along the dermal-epidermal junction, especially in pemphigus erythematosus, now considered to be a localized form of pemphigus foliaceus (Fig. 4.3). It seems that these granules consist of IgG directed against the ectodomain of desmoglein 1, which is shed along the epidermal basement membrane.

In most cases of pemphigus the ECS deposition consists of IgG with or without complement binding, although in some cases also IgA is present. However, in rare cases only IgA depositions can be found, a so-called IgA pemphigus (see Chap. 11). In general two variants of IgA pemphigus are considered, the subcorneal pustulosis type and the intraepidermal neutrophilic type. Direct immunofluorescence of the subcorneal pustulosis type shows deposits of IgA only in the upper part of the epidermis, while in the intraepidermal neutrophilic type IgA is present on the ECS throughout the entire epidermis.

Paraneoplastic pemphigus is a severe autoimmune multiorgan disease different from pemphigus vulgaris [5]. It is characterized clinically by painful stomatitis and polymorphous cutaneous manifestations in patients with underlying neoplasia. PNP comprises many antibodies; the most characteristic are periplakin and envoplakin next to desmoglein. *Paraneoplastic pemphigus is a severe multiorgan disease with in almost all cases antibodies against envoplakin and periplakin.* Direct immunofluorescence shows ECS deposits of IgG and complement throughout the epidermis consistent with other variants of pemphigus. In addition, in some cases a linear deposition of IgG and complement can be seen, which can be attributed to additional antibodies against hemidesmosomal components (Fig. 4.4). However, in these cases the diagnosis of paraneo-

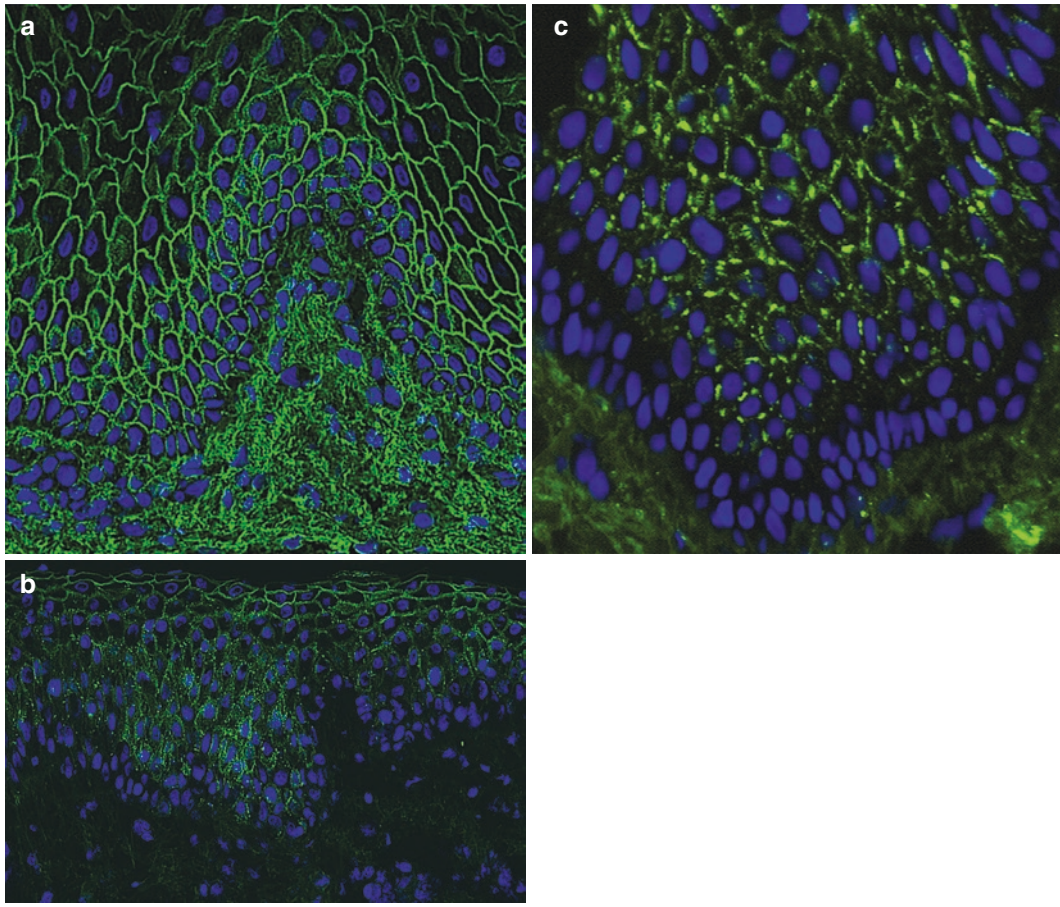

Fig. 4.1 Patterns of epithelial cell surface (ECS) staining in pemphigus: (**a**) smooth pattern, (**b**) fine granular pattern, (**c**) coarse granular pattern

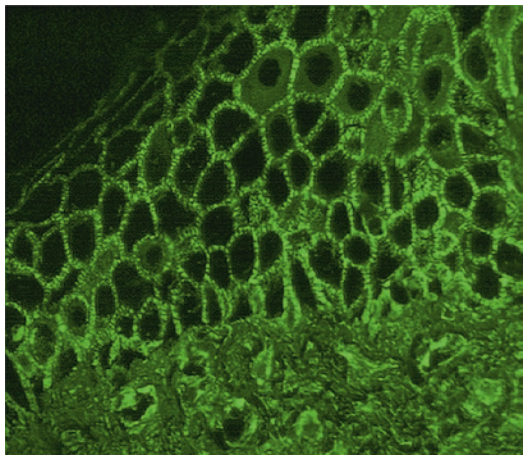

Fig. 4.2 Fals-positive pseudo-epithelial cell surface (ECS) staining of IgG in a tram rail pattern in eczema due to spongiotic edema

plastic pemphigus has to be confirmed by serology, since rare cases of coexisting pemphigus and pemphigoid are described in literature.

Pemphigoid

All variants of are characterized by a linear deposition of immunoglobulins and/or complement along the epidermal basement membrane zone [6] (Fig. 4.5a). *Pemphigoid is characterized by a linear deposition of immunoreactants along the basement membrane* These antibodies are directed against various hemidesmosomal components and connecting molecules; (1) type XVII collagen (BP180) in bullous pemphigoid (BP), mucous membrane pemphigoid (MMP), pemphi-

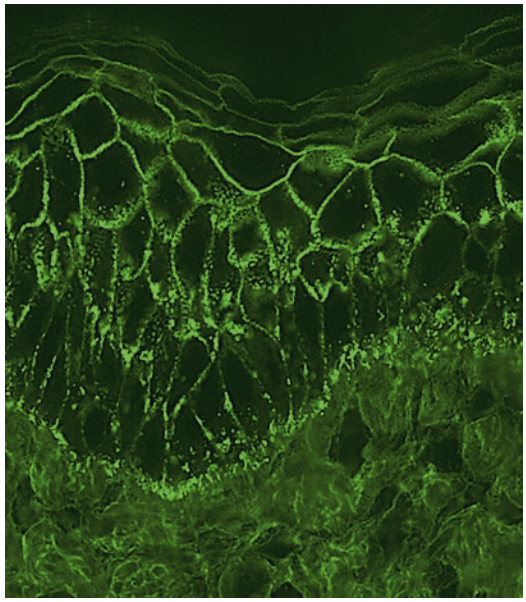

Fig. 4.3 Pemphigus erythematosus with IgG in a smooth/granular ECS deposition, and additionally a granular deposition along the epidermal basement membrane zone

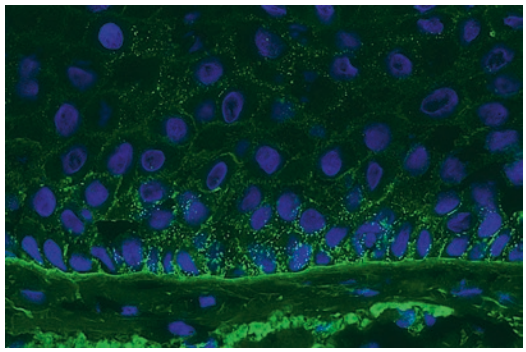

Fig. 4.4 Paraneoplastic pemphigus with IgG in a granular ECS deposition and additionally a linear deposition along the epidermal basement membrane zone

goid gestationis (PG), lichen planus pemphigoides (LPP), and linear IgA disease (LAD), (2) BP230 in BP, (3) laminin 332 in anti-laminin 332 pemphigoid, (4) integrin beta4 in ocular mucous membrane pemphigoid, and (5) p200 in anti-p200 pemphigoid. Moreover, in epidermolysis bullosa acquisita (EBA) and bullous SLE, antibodies against type VII collagen, present in the sublamina densa, also give rise to a linear deposition pattern.

In case a linear deposition is observed it is important to determine the nature of the deposits. In most variants of BP and in EBA the deposits consist of IgG and complement. Mixed IgG/IgA depositions are commonly encountered, especially in mucosal dominant cases of pemphigoid. In addition, in some cases only IgA is present, leading to a diagnosis of LAD or IgA EBA [7, 8]. However, in mucosal dominant pemphigoid with mixed IgA/IgG depositions, the IgG component might be very weak, which might result in a misdiagnosis of linear IgA disease. PG shows in virtually all cases a strong linear deposition of complement along the basement membrane with a weaker staining for IgG. Strikingly, in many cases of PG interruptions in this linear deposition can be seen, caused by the presence of melanocytes (Fig. 4.5b). This can also been seen in other cases of pemphigoid, but is usually less obvious. Although in a number of cases linear IgM deposition might be present in adjunct to IgG and complement, cases have been described with only linear IgM deposition. Whether these cases should be considered a variant of pemphigoid or merely a coincident finding is unknown.

In LPP, clinically characterized by blisters next to typical lichen planus lesions, in addition to a linear IgG deposition, shaggy deposition of fibrin and lichenoid infiltrate is often found (Fig. 4.5c). Furthermore, colloid bodies, ovoid or round structures consisting of keratin filaments and covered with immunoglobulins, can be found in the underlying dermis.

Bullous SLE is characterized by antibodies to type VII collagen in a patient fulfilling the ARA criteria for systemic lupus erythematosus. In bullous SLE, next to or superimposed on a linear IgG deposition, a biopsy might show a lupusband, characterized by granular deposition of immunoglobulins and complement, and the presence of epidermal *in vivo* anti-nuclear antibodies.

In most cases of pemphigoid a linear-serrated pattern can be discerned. This serration pattern can be separated in an n-serrated pattern and a u-serrated pattern [9] (Fig. 4.6a, b). *Bullous pemphigoid shows an n-serrated pattern,*

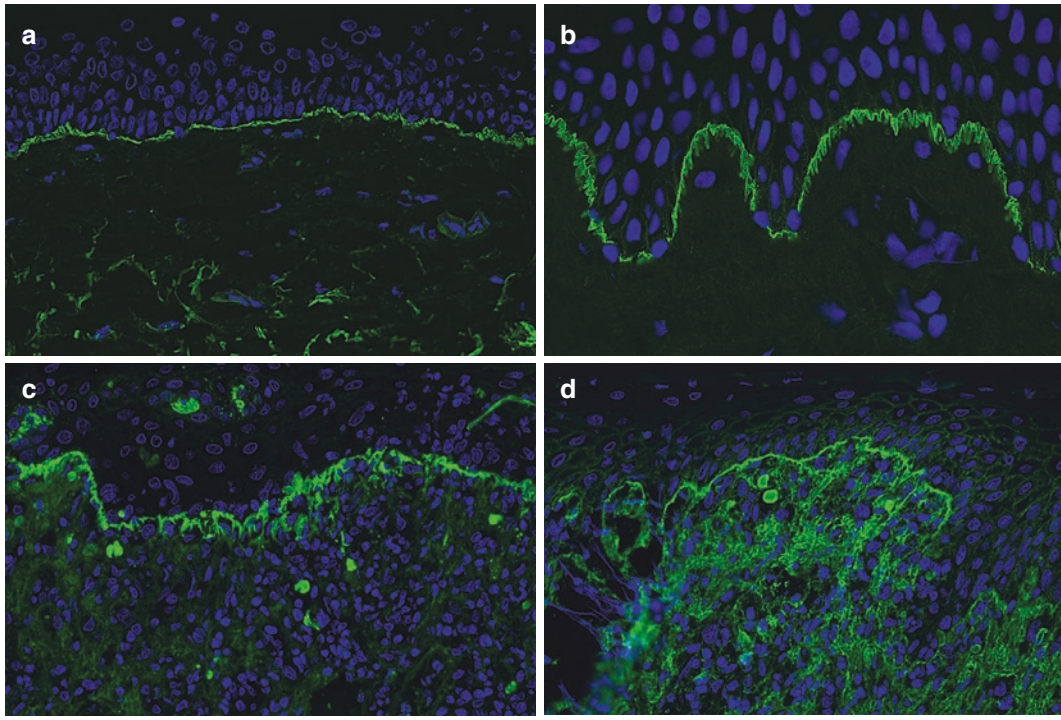

Fig. 4.5 (**a**) Linear deposition of IgG along the basement membrane in bullous pemphigoid. (**b**) Linear deposition of complement with marked gaps due to the presence of melanocytes in pemphigoid gestationis. Shaggy deposition of fibrin (**c**) and a linear deposition of IgG (**d**) along the epidermal BMZ in lichen planus pemphigoides

whereas epidermolysis bullosa acquisita shows a u-serrated pattern. The recognition of these serration patterns makes it possible to differentiate between (1) sublamina densa binding diseases caused by autoantibodies against type VII collagen, e.g. EBA and bullous SLE and (2) blistering diseases with binding above the lamina densa with antibodies against hemidesmosomal components, e.g. BP, PG, MMP, anti-p200 pemphigoid, and anti-laminin 332 pemphigoid. This differentiation can be explained by the fact that in cases with antibodies against type VII collagen the immunodeposits are located between the rootlets of the basal keratinocytes, leading to a u-serrated pattern (Fig. 4.6c). On the other hand, depositions above the lamina densa follow the plasma membrane in the basal cell rootlets, resulting in an n-serrated pattern (Fig. 4.6d). In some cases it is not possible to determine the serration pattern, especially in mucosal biopsies. In these cases it is wise to cut thinner sections or to take a biopsy of clinically uninvolved skin.

However, a few cases remain in which it is impossible to differentiate between an n-serrated and an u-serrated pattern. In these cases the level of the deposition of the antibodies can be determined by fluorescent overlay antigen mapping (FOAM). FOAM is a technique based on the possibility to visualize a targeted antigen relative to a topographic marker. For instance, in Fig. 4.6 red staining is used for type VII collagen, as topographic reference marker and a green staining for IgG deposits. In case of BP separate patterns of IgG deposits (green) and type VII collagen (red) can be seen with red staining on the dermal side. In contrast, EBA skin shows a pattern with overlap of green IgG deposits and red type VII collagen staining, resulting in a yellow-orange fluorescence and lacking red staining on the dermal side. FOAM can be done using a standard immunofluorescence microscope, providing appropriate software is available. However, better results are accomplished using confocal microscopy.

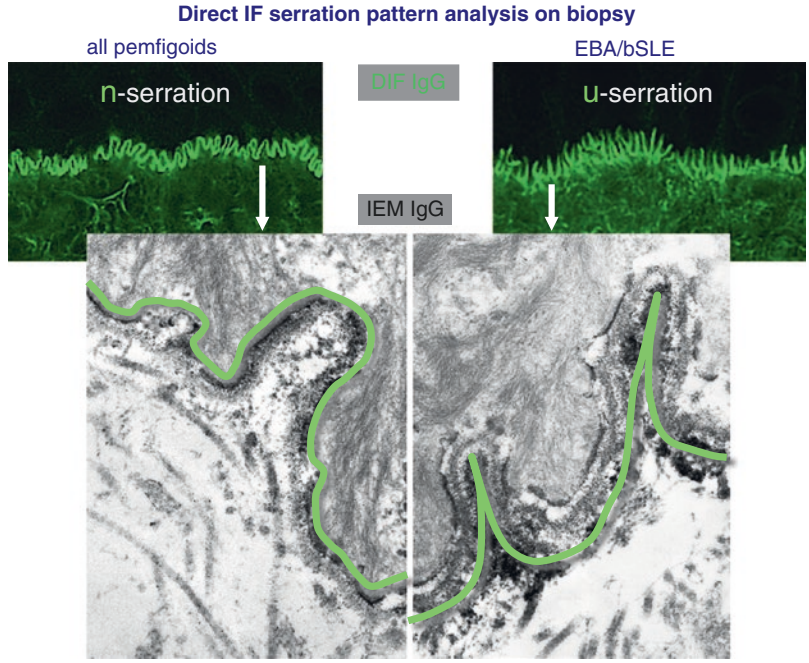

Fig. 4.6 (**a**) n-serrated pattern in bullous pemphigoid, (**b**) u-serrated pattern in epidermolysis bullosa acquisita, (**c–d**) immunoelectron microscopy of peroxidase labeled IgG of perilesional skin from a patient with bullous pemphigoid (**c**) and epidermolysis bullosa acquisita (**d**). The n-serrated pattern follows the undulations of the plasma membrane, whereas the u-serrated pattern is the result of staining of anchoring fibrils between the rootlets. [Reprinted from: Vodegel, R. M., Jonkman, M. F., Pas, H. H. & de Jong, M. C. J. M. U-serrated immunodeposition pattern differentiates type VII collagen targeting bullous diseases from other subepidermal bullous autoimmune diseases. Br. J. Dermatol. 151, 112–118 (2004), with permission from Wiley.]

Dermatitis Herpetiformis

Dermatitis herpetiformis (DH) is characterized by IgA antibodies against tissue transglutaminase and although it has typical pruritic blisters on predilection sites, the clinical picture might resemble various variants of pemphigoid. However, direct immunofluorescence can make a clear distinction between these entities. Direct immunofluorescence of DH shows a granular deposition of IgA along the dermal-epidermal junction [10]. *Dermatitis herpetiformis is characterized by a granular deposition of IgA along the dermal-epidermal junction.* Typically, these depositions are concentrated in the dermal papillae, although in many cases a linear granular is present (Fig. 4.7). This deposition is most probably the result of the precipitation of IgA antibodies against epidermal transglutaminase (TG3). These IgA-TG3 immune complexes can also be detected in small vessels in the papillary dermis.

Porphyria Cutanea Tarda and Pseudoporphyria

Porphyria cutanea tarda (PCT) is characterized by cell poor blisters mostly present on the dorsal sites of hand and feet, induced by photosensitization of endogenous (porphyrins) or exogenous (e.g. NSAID's) agents. Although this disease shows a typical clinical presentation and has a characteristic histology, the differentiation from mechanobullous EBA can be difficult. Fortunately, both entities have different immunofluorescent patterns. As described above EBA is characterized by u-serrated linear deposition of IgG and complement along the basement membrane. PCT, on the other hand,

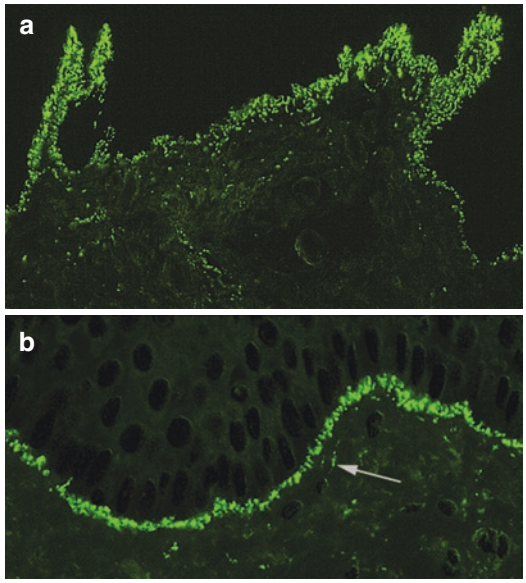

Fig. 4.7 (**a**) The granular IgA depositions in dermatitis herpetiformis are located in the dermal papillae, or (**b**) more along the dermal-epidermal junction and in superficial vessel walls (arrows)

shows a homogeneous deposition of immunoglobulins, preferably IgG, in vessel walls and in most instances a homogeneous deposition along the dermal-epidermal junction (Fig. 4.8), although also granular and fibrillar depositions have been described. *A homogeneous deposition of particularly IgG along the dermal-epidermal junction and in vessel walls is typical in porphyria* It has been hypothesized that the depositions in the vessel walls might reflect a reaction between physiological autoantibodies and damaged vascular endothelium. The formation of separation at the lamina lucida is a secondary event caused by the release of proteolytic enzymes and destruction of laminin and type IV collagen.

Review Questions

1. Pemphigus is characterized by
   a. A smooth epithelial surface staining
   b. A granular epithelial surface staining
   c. Both patterns can be observed

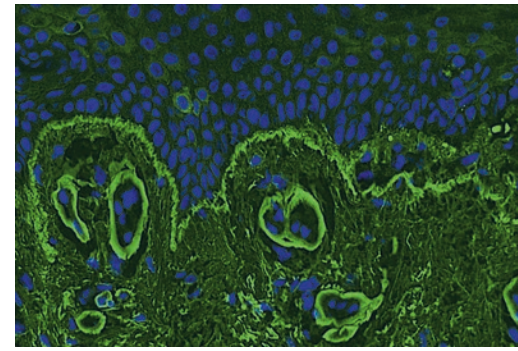

Fig. 4.8 Homogeneous deposition of IgG along the dermal-epidermal junction and in vessel walls is the hallmark of (pseudo)porphyria

2. An u-serrated linear staining along the basal membrane zone can be observed in
   a. Bullous pemphigoid
   b. Epidermolysis bullosa acquisita
   c. Anti-p200 pemphigoid

Answers

1. c
2. b

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