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Chapter 7

Histomorphologic and Direct Immunofluorescence Findings of Autoimmune Bullous Disease

Deniz Ates

Abstract

Autoimmune bullous diseases mainly present with vesiculobullous reaction pattern. First algorithm to approach skin biopsy of autoimmune vesiculobullous disease should be localization of the anatomic level of the split, which could be either intraepidermal or subepidermal. Second, inflammatory cell component should be evaluated, which could vary due to age of the lesion. Third, presence or absence of acantholysis should be considered. Finally, pattern of positive immunofluorescence results or negativity helps to render definitive diagnosis. In this chapter, practical histopathology and direct immunofluorescence findings of autoimmune bullous disease are discussed and supported by illustrative microphotographs taken from cases within our institution.

Keywords: histology, histopathology, autoimmune bullous disease, immunofluorescence, autoimmune vesiculobullous disease

1. Introduction

Autoimmune bullous diseases can be debilitating or even fatal. Therefore, disease control is crucial, and this cannot be achieved without a definitive diagnosis. One of the most important diagnostic tools is light microscopic findings with the additional support of immunofluorescence results. Furthermore, vesiculobullous reaction patterns can be seen in various dermatologic conditions. Histopathological assessment with clinical correlation is also important in ruling out nonautoimmune blistering disease.

To render a definitive result, the histomorphologist follows an algorithm starting with the exclusion of nonautoimmune blistering diseases with morphological and clinical findings.
Second, the anatomic level of the split should be localized, which could be either intraepidermal or subepidermal. Third, the predominant inflammatory cell component should be determined, which can vary due to age of the lesion. Then, additional pathologic features, such as presence or absence of acantholysis, should be considered. The combination of all clinical and histomorphological data with results of immunofluorescence assays and salt split tests is crucial for diagnosing autoimmune vesiculobullous diseases.

In this chapter, practical histomorphological and direct immunofluorescence findings on autoimmune blistering skin diseases are explained using illustrative microscopic photographs. Autoimmune bullous diseases are classified as follows:

1. Pemphigus group
   a. Pemphigus vulgaris
   b. Pemphigus vegetans
   c. Pemphigus foliaceus
   d. Pemphigus erythematosus
   e. Endemic pemphigus
   f. IgA pemphigus
   g. Pemphigus herpetiformis
   h. Paraneoplastic pemphigus
   i. Drug-induced pemphigus

2. Subepidermal autoimmune bullous disease
   a. Subepidermal blisters with eosinophils
      i. Bullous pemphigoid
      ii. Gestational pemphigoid
   b. Subepidermal autoimmune bullous diseases with neutrophils
      i. Dermatitis herpetiformis
      ii. Linear IgA bullous dermatitis
      iii. Mucous membrane (cicatricial) pemphigoid
      iv. Anti-p200 pemphigoid
      v. Bullous lupus erythematosus
      vi. Epidermolysis bullosa acquisita
2. Pemphigus group

2.1. Pemphigus vulgaris

Pemphigus vulgaris is a suprabasilar acantholytic vesiculobullous disease. Extension of a cleft formation throughout the adnexal epithelium is its characteristic. Basal cells lose their intercellular connections but retain dermal attachments. Hence, they attain their classical "tombstone" appearance (Figure 1). Split space usually contains detached acantholytic cells. Occasionally, a few eosinophils and neutrophils accompany acantholytic cells in the bulla cavity.

The early stages of pemphigus vulgaris, epidermal edema and intercellular bridges are lost at the epidermis and adnexal epithelium. One should be alerted for follicular acantholysis because it could be a clue for early diagnosis for pemphigus vulgaris. Early lesion may show eosinophilic spongiosis.

Figure 1. Pemphigus vulgaris. (a) Subrabasal split with few acantholytic cells (40×). (b) Classical "tombstone" appearance at the basal layer (100×). (c) Extention of subrabasal split throughout the adnexal epithelium (200×). (d) Intraepidermal IgG positivity mainly at the deep levels of the epidermis (400×).
The dermal level exhibits mild nonspecific changes such as mild perivascular dermatitis with mild mix inflammation usually containing eosinophils.

A biopsy should be taken from nonaffected perilesional area. Direct immunofluorescence shows intraepidermal intercellular IgG deposition predominantly in the lower portion (Figure 1). Less frequently, C3, IgM and IgA intercellular positivity is present. Outer root sheet of the anagen hair follicle may also be positive within intercellular area.

Application of C4d immunohistochemistry on routinely processed tissue sections could be a helpful tool for pemphigus vulgaris [1].

2.2. Pemphigus vegetans

Pemphigus vegetans is characterized by a vegetative histologic appearance caused by hyperkeratosis, acanthosis, papillomatosis and downward proliferation of rete ridges. On occasion, proliferation can be exuberant condition known as “pseudoepitheliomatous hyperplasia.” Epidermal hyperplasia involves both the follicular epithelium and the epidermis. Characteristically, mild suprabasilar acantholysis and an intense collection of inflammatory infiltrate with neutrophils and eosinophils are present. The dermis contains a heavy infiltrate of lymphocytes, eosinophils and/or neutrophils.

Direct immunofluorescence findings are the same as those of pemphigus vulgaris, as both show intercellular deposition of IgG and/or C3.

2.3. Pemphigus foliaceus

Bullas of Pemphigus foliaceus are very fragile due to superficial splitting, thus, it is very difficult to obtain intact bulla. Biopsy taken from an established lesion reveals an upper granular or subcorneal split (Figure 2). In the bulla cavity, it is common to see acantholytic cells, fibrin and some neutrophils. Through careful examination, a pathologist can detect a focal acantholysis inside the follicular epithelium. When the bulla cavity contains neutrophils, other subcorneal blistering disorders (such as bullous impetigo, staphylococcal scaled skin syndrome, IgA pemphigus and subcorneal pustular dermatosis) will be in differential. Immunofluorescence will be helpful in making this distinction.

The superficial dermis could be edematous with a mixed inflammatory cell infiltrate.

There are some uncommon histologic features of pemphigus foliaceus, which are presented in Table 1.

Late lesions can show parakeratosis and acanthosis. Dyskeratotic cells resembling Darier’s corps and rounds can accompany.

Direct immunofluorescence shows intercellular staining of IgG and C3 mostly at the higher levels of the epidermis.

Immunofluorescence is a helpful tool to differentiate other lichenoid-looking lesions in oral mucosa, and it is recommended to perform when a lichenoid lesion is detected [2].
2.4. Pemphigus erythematosus

The H&E appearance of pemphigus erythematosus is almost the same as that of the pemphigus foliaceus (an upper granular or subcorneal split with acantholysis).

Table 1. Uncommon histologic features and more related disease of pemphigus foliaceus.

<table>
<thead>
<tr>
<th>Uncommon histological manifestation</th>
<th>More related disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophilic spongiosis</td>
<td>IgA pemphigus</td>
</tr>
<tr>
<td>Neutrophilic pustules</td>
<td>IgA pemphigus</td>
</tr>
<tr>
<td></td>
<td>Pustular psoriasis</td>
</tr>
<tr>
<td></td>
<td>Bullous impetigo</td>
</tr>
<tr>
<td></td>
<td>Subcorneal pustular dermatosis</td>
</tr>
<tr>
<td>Eosinophilic spongiosis</td>
<td>Endemic pemphigus</td>
</tr>
</tbody>
</table>

Figure 2. Pemphigus foliaceus. (a) Upper granular split (40×). (b) Fragile bulla is tearing off the upper epidermal layers (40×). (c) Acantholytic granular layer cells (200×). (d) Intraepidermal IgG positivity mainly at the superficial levels of the epidermis (200×).

2.4. Pemphigus erythematosus

The H&E appearance of pemphigus erythematosus is almost the same as that of the pemphigus foliaceus (an upper granular or subcorneal split with acantholysis).
Direct immunofluorescence displays intercellular and dermoepidermal linear positivity of IgG and/or C3. The dermoepidermal positivity of IgG could be related to sun exposure [3].

2.5. Endemic pemphigus foliaceus (fogo selvagem)

Histological features are very similar to those of pemphigus foliaceus; a superficial split can be seen if non-bullous erosions are not biopsied. Early lesions typically show eosinophilic spongiosis. An established lesion can display inflammatory cells (mainly neutrophils) and a few acantholytic cells.

2.6. IgA pemphigus

Two major types are identified:

- Subcorneal pustular dermatosis (SPD) (IgA pemphigus foliaceus).
- Intraepidermal neutrophilic IgA dermatosis (IEN) (IgA pemphigus vulgaris).

In the SPD variant, vesicles are typically located at a subcorneal location. The bulla cavity is usually full of neutrophils (Figure 3). In the IEN, pustules can be found throughout all layers of the epidermis. Hair follicle involvement is also evident. Apart from the major two subtypes, patients with IgA pemphigus can show histomorphological features of P. vulgaris, P. foliaceus or pemphigus vegetans [4].

Direct immunofluorescence exhibits intraepidermal IgA positivity. SPD variant IgA deposition is at the superficial layers of epidermis, whereas in IEN variant fluorescence, it is seen throughout the epidermis.

2.7. Pemphigus herpetiformis

Inflammatory cell infiltrate (eosinophils, neutrophils or both) and acantholysis are usually prominent. Subcorneal or intraepidermal eosinophilic/spongiotic abscess formation is frequent. Pemphigus herpetiformis histomorphology usually presents as eosinophilic spongiosis.

![Figure 3. IgA pemphigus. (a) Intra/sub corneal vesicle (40×). (b) The bulla cavity is full of neutrophils (200×).](image_url)
Direct immunofluorescence usually demonstrates IgG and/or C3 in the intercellular spaces [5].

2.8. Paraneoplastic pemphigus

The histomorphological findings of paraneoplastic pemphigus are highly variable. Characteristically:

1. Suprabasal acantholysis with cleft of vesicle formation (resembles pemphigus foliaceus)
2. Interphase changes dense lymphohistiocytic infiltrate with basal degeneration and dyskeratotic cells
3. Spongiosis and lymphocyte exocytosis
4. Pigment incontinence is in evidence
5. Eosinophils are rare.

Some studies show that keratinocyte necrosis is associated with an adverse prognosis [3]. Direct immunofluorescence shows intercellular and linear basement membrane staining with C3 and IgG.

2.9. Drug-induced pemphigus

Subcorneal split, spongiosis with eosinophils, necrotic foci of keratinocytes, focal acantholysis are mentioned as histomorphologic findings of drug-induced pemphigus [6]. Unfortunately, drug-induced pemphigus is indistinguishable from idiopathic counterparts based on histomorphological and immunofluorescence findings [4].

Notably, vesiculobullous reaction patterns can be seen in many other dermatologic conditions. Histomorphologic evaluation should begin with exclusion of other diseases if possible and should consider other histologic features.

Nonautoimmune vesiculobullous diseases with subcorneal and intraepidermal split are given in Table 2.

<table>
<thead>
<tr>
<th>Intracorneal and subcorneal blisters</th>
<th>Intraepidermal blisters</th>
<th>Suprabasilar blisters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Impetigo</td>
<td>Spongiotic blistering diseases</td>
<td>Hailey-Hailey</td>
</tr>
<tr>
<td>Staphlococcal “scalded skin” syndrome</td>
<td>Palmoplantar pustulosis</td>
<td>Darier’s disease</td>
</tr>
<tr>
<td>Dermatophytosis</td>
<td>Erosive pustular dermatosis of the leg</td>
<td>Grover’s disease</td>
</tr>
<tr>
<td>Subcorneal Pustular Dermatosis</td>
<td>Viral blistering Disease</td>
<td>Acantholytic solar keratosis</td>
</tr>
<tr>
<td>Infantile Acropustulosis</td>
<td>Friction Blister</td>
<td></td>
</tr>
<tr>
<td>Erythema toxicum neonatorum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transient Neonatal Pustular Melanosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute Generalized Exanthematous Pustulosis</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Non-autoimmune vesiculobullous diseases with subcorneal and intraepidermal split.
3. Subepidermal bullous disease

3.1. Subepidermal diseases with predominantly eosinophils

3.1.1. Bullous pemphigoid

A well-developed bullous pemphigoid blister is classically subepidermally located and is unilocular (Figure 4). Inflammatory cells are seen in the blister cavity, predominantly eosinophils. The dermal papillary outline is typically retained, and papillary dermal bulges are typically projected into the bulla cavity otherwise known as festooning. Bullous pemphigoid histological findings vary according to the duration of the lesion and the clinical appearance of the biopsy site.

If the biopsy is taken from apparently normal skin, a sparse dermal infiltrate is seen. However, when a biopsy is taken from a lesion with an erythematous base, a more prominent dermal infiltrate is seen sometimes accompanied by eosinophilic spongiosis. It is not unusual to see an eosinophilic flame in clinically erythematous-based lesions.

![Figure 4. Bullous pemphigoid. (a) Unilocular subepidermal located split (40×). (b) Dermal papillary outline is typically retained (100×). (c) Predominantly eosinophilic dermal inflammatory infiltrate is seen (200×). (d) Homogenous linear positivity with IgG at the basal membrane zone.](image)
In lesions of several days duration, the blister may appear intraepidermal.

Prodromal lesions can show edema of the papillary dermis and a superficial perivascular dermatitis with eosinophils, neutrophils and lymphocytes is usually accompanied by an inflammatory component (Figure 5). An eosinophilic spongiosis pattern is also a well-known feature of early lesions of bullous pemphigoid. Occasionally, bullous pemphigoid lesions are accompanied by a predominance of neutrophils and aligned close to the basilar layer portion of the papillary dermis.

When direct immunofluorescence is applied to the biopsy from the perilesional area, it shows homogenous linear positivity with IgG and/or C3 at the basal membrane zone. Prodromal lesions may be positive with C3 only. IgA and IgM are positive in 20% of the cases [7].

Application of C4d immunohistochemistry on routinely processed tissue sections could be a helpful tool for bullous pemphigoid and confirms immunoreactant deposition [1, 8].

3.1.2. Gestational pemphigoid

A biopsy taken from an early lesion often shows the prominent dermal edema looking like a “teardrop.” The edema consists of various inflammatory cells predominantly eosinophils. Tips of the dermal papilla consist of spongiosis and/or necrotic keratinocytes. The dermis underlying vesicle demonstrates a perivascular and interstitial inflammatory infiltrate mainly of eosinophils. Neutrophils, histiocytes and lymphocytes are the accompanying cell component. Neutrophils may also predominate.

Direct immunofluorescence will show C3 positivity, and 30–50% of cases show linear IgG positivity at the basal membrane zone [4].

Microscopic differential diagnosis in the early stages can include urticarial reactions and conditions associated with eosinophilic spongiosis. Without any clinical information, classical lesions are almost impossible to differentiate from bullous pemphigoid.
3.2. Subepidermal diseases with predominantly neutrophils

3.2.1. Dermatitis herpetiformis (Duhring disease)

Biopsies taken from established bulla show subepidermal blisters with a dermal papillary neutrophilic microabscess, which is a hallmark histologic feature of dermatitis herpetiformis (Figure 6). Fibrin found at the tips of the dermal papilla forms a reticular network at the bulla cavity. When the duration of the lesion reaches to 38–48 h, neutrophilic consistency of bulla cavity decreases and eosinophils increase. The roof of the bulla cavity has usually smooth outline, contrast to the “festooning” pattern of the bullous pemphigoid.

At the dermis, mixed inflammatory cell infiltrate consists of abundant neutrophils. Leukocytoclasis and swelling of the endothelium are typical findings; however, vasculitis is not in evidence.

Elementary lesions of celiac disease in an intestinal mucosal biopsy occur due to an increase in intraepidermal lymphocytes and crypt hyperplasia and a decrease in villous height. These histological changes are reversed following a gluten-free diet.

Figure 6. Dermatitis Herpetiformis. (a) Subepidermal split with reticular fibrin network (100×). (b) Dermal papillary microabcess (100×). (c) Eosinophilic consistency of the lesion (200×). (d) Granular IgA deposition in the dermal papillae (200×).
Direct skin immunofluorescence shows granular IgA deposition in the dermal papillae.

The histological differential diagnosis of dermatitis herpetiformis includes linear IgA disease, bullous pemphigoid and bullous lupus. Immunofluorescent studies are critical in making this distinction.

3.2.2. Linear IgA dermatosis

Linear IgA light microscopic features are subepidermal blisters with neutrophils; these frequently resemble dermatitis herpetiformis. The neutrophilic infiltrate is more localized to dermal papilla in dermatitis herpetiformis, whereas in linear IgA dermatosis neutrophils are more widespread. On occasion, eosinophils are also seen in the bulla cavity. The disease is rarely presented with eosinophilic spongiosis.

Direct immunofluorescence reveals homogenous linear IgA deposition along the basement membrane. In 80% of the cases, IgA is the only immunoreactant. IgG, IgM and C3 may also be present.

3.2.3. Mucous membrane pemphigoid

The mucous membrane pemphigoid consists of a varying number and consistency of inflammatory cells, depending on the age of the lesion. Lesions of less than 48 h duration, like dermatitis herpetiformis, present with dermal papillary microabscesses. As a lesion gets older, the neutrophil consistency of the infiltrate decreases and the eosinophil and latter lymphocyte content increases. Even in early lesions, if the biopsy corresponds to a previous bulla site, mucosal scar formation is in evidence.

The dermis contains perivascular lymphohistiocytic infiltrate accompanied by neutrophils, eosinophils and plasma cells. In late lesions, the superficial dermis shows scarring, with or without subepidermal split.

Direct immunofluorescence shows linear deposits of IgG and often C3 along the basement membrane. Positivity rate increases in buccal mucosa.

3.2.4. Anti p200 pemphigoid

It is a very rare subepidermal blistering disease. A study of 12 cases by Meijer JM et al. in 2014 found that subepidermal blistering is present in every case. An upper dermal infiltrate of eosinophils and neutrophils was seen in nearly all cases. Direct immunofluorescence results displayed IgG and C3 positivity in all cases [9].

3.2.5. Bullous lupus erythematosus

In bullous systemic lupus erythematosus, the findings are often identical to dermatitis herpetiformis. The blister cavity consists of fibrin and many neutrophils. Lymphocytes, histiocytes and eosinophils are occasionally seen. Nuclear dusts (leukocytoclastic debris) are evident in both bulla cavity and perivascular areas. Perivascular neutrophilic debris and the evidence of
vasculitis usually situated deeper than dermatitis herpetiformis. Vacuolar interphase change with dyskeratotic cells is occasional.

Classically, by using direct immunofluorescent test, the disease is characterized by the presence of IgG and C3 at the basement membrane. Pattern could be both linear and granular. Biopsy infrequently shows epidermal nuclear IgG staining, which is diagnostically very helpful staining. Concurrence of IgG, IgA and IgM positivity is not rare. Immunoreactants are also demonstrated at the vessel walls.

3.2.6. Epidermolysis bullosa acquisita

Classical pattern is a cell-free subepidermal blister. Disease can present with neutrophil (papillary microabscesses) or occasionally, eosinophil-rich infiltrate. Blister roof is usually intact, although some dermal fragments and fibrin may be displayed. PAS positivity is demonstrated at the bulla roof due to the basement membrane split.

Direct immunofluorescence shows linear deposition immunoglobulins particularly, IgG and C3 along the basement membrane.

Nonautoimmune vesiculobullous diseases with subepidermal split should always be in differential (Table 3).

<table>
<thead>
<tr>
<th>Subepidermal blisters with little inflammation</th>
<th>Subepidermal blisters with lymphocytes</th>
<th>Subepidermal blisters with eosinophils</th>
<th>Subepidermal blisters with neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porphyria cutanea tarda</td>
<td>Erythema multiforme</td>
<td>Arthropod bite</td>
<td>Bullous urticaria</td>
</tr>
<tr>
<td>Burns and cryotherapy</td>
<td>Paraneoplastic pemphigus</td>
<td>Drug reactions</td>
<td>Bullous acute vasculitis</td>
</tr>
<tr>
<td>Toxic epidermal necrolysis</td>
<td>Fixed drug eruption</td>
<td>Epidermolysis bullosa</td>
<td>Erysipelas</td>
</tr>
<tr>
<td>Suction blisters</td>
<td>Lichen sclerosus Et</td>
<td></td>
<td>Sweet syndrome</td>
</tr>
<tr>
<td></td>
<td>atrophicus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blister overlying scars</td>
<td>Lichen planus pemphigoides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bullous solar elastosis</td>
<td>Polymorphic light eruption</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bullous amyloidosis</td>
<td>Bullous lichen planus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bullous drug reaction</td>
<td>Bullous allergic contact dermatitis</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Non-autoimmune vesiculobullous diseases with subepidermal split.

4. Salt split testing

This is a modified indirect immunofluorescence technique that aims to produce artificial split between lamina lucida and lamina densa of the basal membrane. The split roof is supposed to be covered by lamina lucida and the floor by lamina densa. Artificial splitting is obtained by
treating normal skin with a 10–15 ml 1 M NaCl solution for 48 h at 4°C. The direct technique then involves applying IgG to the tissue. Split testing with a saline application is commonly used in daily practice. Practical use of salt split test is summarized in Table 4.

Practical summary of histomorphology and immunofluorescent findings of autoimmune vesiculobullous diseases is given in Table 5.

<table>
<thead>
<tr>
<th>Disease with supralamina densa split (roof labeling)</th>
<th>Disease with sublamina densa split (floor labeling)</th>
<th>Variable diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bullous pemphigoid</td>
<td>Epidermolysis bullosa acquisita</td>
<td>Linear IgA</td>
</tr>
<tr>
<td>Pemphigoid gestationis</td>
<td>Bullous systemic lupus erythematosus</td>
<td>Mucosal pemphigoid (mostly supralamina densa-roof)</td>
</tr>
</tbody>
</table>

Table 4. Practical use of salt split technique in autoimmune vesiculobullous diseases.

<table>
<thead>
<tr>
<th>Autoimmune disease</th>
<th>Target antigen</th>
<th>Anatomic localization and pattern of immunofluorescence</th>
<th>Anatomic localization of split</th>
<th>Staining of salt split test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pemphigus vulgaris</td>
<td>Desmoglein-3</td>
<td>IgG and/or C3. Intraepidermal – predominately basal</td>
<td>Suprabasal</td>
<td>N/A</td>
</tr>
<tr>
<td>Bullous pemphigoid</td>
<td>BPAg1, BPAg2</td>
<td>IgG and/or C3. Subepidermal - linear</td>
<td>Subepidermal</td>
<td>Roof</td>
</tr>
<tr>
<td>Dermatitis herpetiformis</td>
<td>Tissue transglutaminase</td>
<td>Granular IgA at the dermoepidermal junction</td>
<td>Subepidermal</td>
<td>N/A</td>
</tr>
<tr>
<td>Linear IgA bullous dermatitis</td>
<td>LABD97, LAD-1, LAD285</td>
<td>Linear IgA at the dermoepidermal junction</td>
<td>Subepidermal</td>
<td>Roof or floor or both</td>
</tr>
<tr>
<td>Bullous lupus erythematosus</td>
<td>Type VII collagen</td>
<td>Linear IgG, C3</td>
<td>Subepidermal</td>
<td>Floor</td>
</tr>
<tr>
<td>EBA</td>
<td>Type VII collagen</td>
<td>Linear IgG, C3</td>
<td>Subepidermal</td>
<td>Floor</td>
</tr>
</tbody>
</table>

Table 5. Summary of histomorphology and direct Immunofluorescent findings of autoimmune vesiculobullous diseases.

5. Indirect immunofluorescence

General rules for direct immunofluorescence testing are also valid for indirect immunofluorescence technique. A punch or excisional biopsy from perilesional skin or mucous membrane should be sent to laboratory without fixative within a plastic tube or in isotonic NaCl solution. If the biopsy is planning to be sent elsewhere, biopsy can be placed in a Michel’s solution [10]. This solution is also a well-established medium for polymerase chain reaction analysis [11]. Laboratory method is complicated than the direct method, so it is not preferred in daily routine practice. First, the patient serum should be prepared for testing. The blood sample is
needed to be centrifuged to separate the serum. A total of 4–6 micron thick slides are prepared from the biopsy, then, treated with prepared serum for 30 min. In case of titer requirement, doubling dilution is performed. Slides are washed, and then, the standardized fluorescein-labeled antibody binds to the serum antibody. It is examined under fluorescent microscope.

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