Immunofluorescence Techniques

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INTRODUCTION

Immunofluorescence is a powerful technique that utilizes fluorescent-labeled antibodies to detect specific target antigens. It is used widely in both scientific research and clinical laboratories. This article presents key concepts in the use of antibodies in immunofluorescence and their application in the diagnosis of dermatologic diseases.

ANTIBODIES

An antibody is a protein complex produced by B cells that initiates an immune response against a target antigen. The basic organization of an antibody includes two functional domains that, together, resemble the letter Y (Figure 1, left). The Fab (fragment having the antigen binding site) domain makes up the arms of the Y, and at the end of each arm is a variable region responsible for antigen binding, called the antigen-binding site. The Fc (fragment that crystallizes) domain comprises the tail of the Y, which effector cells, immune proteins, and other antibodies recognize primarily. This unique structure allows direct detection of antigens in the skin using a single fluorophore-labeled antibody or indirect detection through binding of a fluorophore-labeled secondary antibody raised against the Fc domain of an unlabeled primary antibody (Figure 1, right). Because the Fc domain is conserved within a species, the labeled secondary antibody can be used to detect any primary antibody raised from a single species. This system is versatile and cost-effective because few labeled antibodies are required to detect many possible primary antibodies. For more information about laboratory procedures using antibodies, see Harlow and Lane (1999).

In certain bullous diseases, connective-tissue diseases, and vasculitides, patients produce antibodies against an antigen in their own skin or blood vessels. Detection and characterization of the autoantibody–antigen complexes is accomplished by laboratory analysis of a skin biopsy and (possibly) a blood sample. Clinically, it is important to collect the biopsy from the appropriate location for the type of autoimmune disease under consideration. Because immune deposits are degraded in inflamed or blistered skin, bullous diseases require biopsy of normal-appearing skin immediately adjacent to a lesion, whereas connective tissue diseases and vasculitides can be evaluated by biopsy of the skin lesion itself. The biopsy can be stored temporarily in Michel's transport medium (3.12 M ammonium sulfate, 5 mM *N*-ethylmaleimide, 5 mM magnesium sulfate heptahydrate, and 25 mM potassium citrate, pH 7.0) (Michel *et al.*, 1972). The near-saturating concentration of ammonium sulfate preserves autoantibody–antigen complexes of the specimen for days by precipitating the proteins, which prevents them from diffusing away from their original location in the tissue. However, the transport medium is not a fixative, so the integrity of cellular membranes will be lost over time (Vaughn Jones *et al.*, 1995).

WHAT IMMUNOFLUORESCENCE DOES

- Immunofluorescence is a microscope-based technique used clinically to diagnose certain cutaneous diseases by detection of autoantibody–antigen complexes.
- Techniques including direct immunofluorescence, indirect immunofluorescence, and salt-split skin are utilized depending on the clinical scenario.
- Direct immunofluorescence is performed on patients' skin using fluorophore-labeled antibodies that directly bind to the pathogenic autoantibody–antigen complexes in the skin.
- Indirect immunofluorescence techniques are used in dermatology primarily to detect circulating pathogenic autoantibodies.

LIMITATIONS

- Fluorescence signals depend on the quality and concentration of the antibody, proper handling of the specimen, and detection with the appropriate secondary antibodies.

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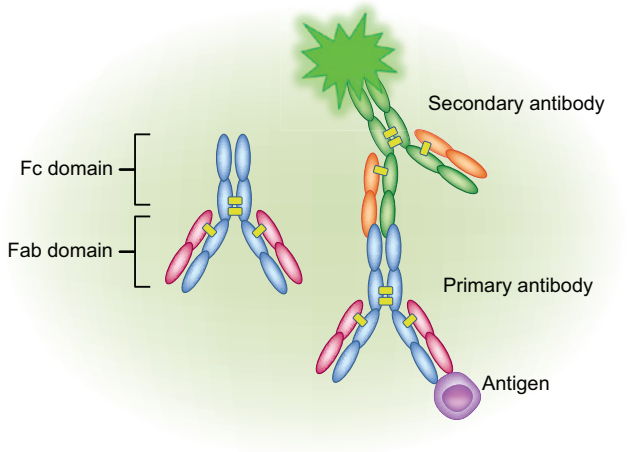

Figure 1. Antibody structure and interactions. (Left) A cartoon of an IgG antibody. Each oval represents a 110-amino-acid domain. The dark blue ovals together represent a heavy-chain polypeptide, the magenta ovals represent a light-chain polypeptide, and the yellow rectangles represent disulfide bonds between polypeptides. (Right) An antigen (purple) and a fluorophore-conjugated secondary antibody demonstrate the binding events between antigen recognition and the fluorescent signal.

DIRECT IMMUNOFLUORESCENCE (DIF)

DIF uses fluorescent-tagged antibodies to bind directly to the target antigen in the skin. In autoimmune bullous diseases, the target antigen is the autoantibody or complement that causes the blister. Once the tissue is received in the laboratory, it is washed, rapidly frozen, and cut into 5- to 6-μm sections that are placed on glass slides. The slides are incubated with the fluorescent-tagged antibodies directed against the target antigen. Typically, a panel of antibodies directed against different antibody isotypes (IgA, IgG, IgM), as well as complement, is used. A drop of each fluorescent-labeled antibody is placed on a separate tissue section as well as appropriate positive and negative controls. The working concentration of each antibody is previously titrated to obtain the highest signal-to-background ratio. The slides are incubated for 30 minutes at room temperature in a dark, humidified chamber. The antibody is then drained, the slides are washed, and the labeled tissue sections are mounted and viewed with a fluorescence microscope. Diagnosis is based on the binding pattern (for example, intercellular, linear at dermal–epidermal junction), as well as the isotype (for example, IgG, IgA) of the antibody detected.

DIF techniques can also be used to detect nonantibody targets in the skin, such as infectious organisms. In this case, a fluorophore-labeled primary antibody directed against the suspected antigen is used to detect the presence or absence of the organism. This technique is rapid and quite specific, but, owing to the limited number of antibodies that can bind to the specific target, it may be less sensitive than other microbiologic techniques.

INDIRECT IMMUNOFLUORESCENCE

Indirect immunofluorescence utilizes a two-step technique, in which a primary, unlabeled antibody binds to the target, after which a fluorophore-labeled second antibody (directed against the Fc portion of the primary antibody) is used to detect the first antibody. This technique is more complicated and time consuming than direct immunofluorescence (because it requires a second incubation period); however, it is more sensitive because more than one secondary antibody can bind to each primary antibody, which amplifies the fluorescence signal. A variation of indirect immunofluorescence testing is used to detect circulating autoantibodies in immunobullous diseases. In this situation, the primary antibody is the suspected autoantibody in the patient serum. The serum is incubated with thin sections of normal human skin or other animal tissues, which are known to consistently and sensitively bind the antibody of interest. Monkey esophagus, for example, is an excellent substrate for detecting antidesmoglein antibodies. When the test is positive, autoantibodies in the serum bind to the target antigen in the tissue sample; the second, fluorophore-labeled antibody then binds to the Fc portion of the autoantibody, allowing it to be visualized with the fluorescence microscope. The fluorescence pattern created by the circulating autoantibodies demonstrates the same pattern as would a perilesional skin biopsy with DIF.

SALT-SPLIT SKIN

Certain diseases cannot be distinguished using routine DIF techniques. Bullous pemphigoid (BP; with autoantibodies against BP180 and BP230 antigens) and epidermolysis

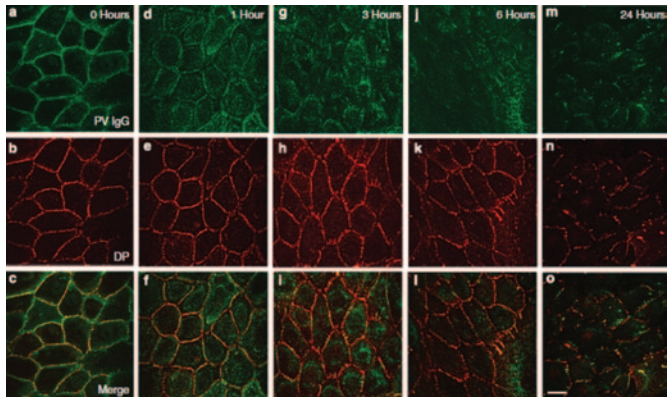

Figure 2. Time course of desmosome disassembly in response to pemphigus vulgaris (PV) IgG. Keratinocytes (KCs) were exposed to PV IgG at 4°C for 20 minutes and subsequently shifted to 37°C for 1, 3, 6 and 24 hours. The localization of human IgG and desmoplakin (DP) was monitored by immunofluorescence microscopy. In cells incubated at 4°C (**a–c**), PV IgG labels cell borders and desmoplakin staining is predominantly in punctate linear patterns at cell-cell junctions. After 1 hour of treatment, with PV IgG (**d–f**), the PV IgG-desmoglein-3 (Dsg3) molecules accumulate in a puncta that are distal to cell-cell borders, whereas desmoplakin (**e**) staining is unchanged. KCs treated with PV IgG for 3 and 6 hours exhibit a rearrangement of desmoplakin into linear arrays emanating from cell borders, which contain both Dsg3 and desmoplakin. Following treatment with PV IgG for 24 hours (**m–o**) both Dsg3 and desmoplakin are noticeably mislocalized and/or absent from cell-cell junctions. Bar = 10 μm. Reprinted from Jennings *et al.* (2011).

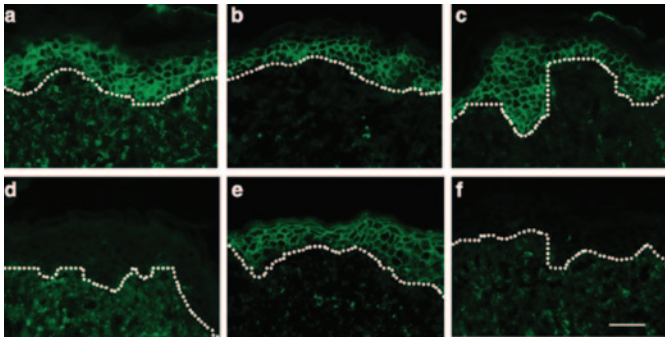

Figure 3. Immunological analysis of anti-desmoglein-3 (Dsg3) mAbs isolated from the paraneoplastic pemphigus (PNP) patient. Indirect immunofluorescence of anti-Dsg3 single-chain variable fragment (scFv) clones in human skin. (**a**) PNP-A1, (**b**) PNP-B1, and (**c**) PNP-C1 showed cell surface staining of the keratinocytes, whereas clone (**d**) PNP-D1, was negative. (**e**) An scFv isolated from a pemphigus vulgaris (PV) patient (D31)2/28 was used as a positive control. (**f**) Pretreatment of human skin cryosections with EDTA prevented the cell binding of PNP-C1 to keratinocytes. Bar = 100 μm. Reprinted from Saleh *et al.* (2012).

bullosa acquisita (with autoantibodies against collagen VII) both create a linear band of IgG and complement at the dermal–epidermal junction. Splitting the skin prior to evaluation allows these target antigens to be more accurately localized. The skin is incubated in 1 M sodium chloride, which splits the skin at the level of the lamina lucida. Collagen VII immune complexes are retained on the dermal side and BP180 immune complexes on the epidermal side of the sample, allowing discrimination between the two diseases.

LIMITATIONS

A number of factors can affect the quality of a fluorescence image. The quality and concentration of the labeled antibody are important to achieve a high signal-to-background ratio. Too much nonspecific antibody binding from either a poor-quality antibody or a high concentration may not allow accurate localization of immune complexes. Alternatively, a dilute antibody may not provide sufficient signal. Proper handling of the biopsy specimen, with storage in Michel's transfer medium or flash freezing, is important to retain the location and antigenicity of the immune complexes. Finally, the signal from fluorescent probes is lost over time with exposure to light (a phenomenon called bleaching). Therefore, it is important to keep samples in the dark when not in use, use only as much light as necessary from the microscope, and use an antifade agent in the mounting medium, such as *p*-phenylenediamine (for a review of the principles of fluorescence microscopy, see Lichtman and Conchello, 2005).

Further limitations of immunofluorescence relate to how dermatologic diseases are diagnosed. Currently, bullous diseases are diagnosed by immunofluorescence based on the location of the autoimmune complexes, the presence of inflammatory infiltrates, the class of the autoantibody, and the morphology of the immune complexes (e.g., continuous, linear, granular) (for a complete review, see Mutasim and Adams, 2001). Because immunofluorescence is the gold standard for diagnosis of bullous diseases, it is difficult to assess the sensitivity and specificity of the technique. Also, if a limited set of antibodies is used in the clinical laboratory, diseases with an untested class of antibody, such as those mediated by IgA, may be missed. This scenario is circumvented by testing all samples with a standardized panel of antibodies directed against human IgG, IgM, and IgA and complement C3. The application of enzyme-linked immunosorbent assay, a technique in which a recombinant antigen is detected with a primary and enzyme-linked secondary antibody, to diagnose autoimmune bullous diseases may have its use in certain clinical settings (Amagai *et al.*, 1999).

IMMUNOFLUORESCENCE IN BULLOUS DISEASES

Jennings *et al.* used immunofluorescence techniques to study the spatial and temporal changes of the desmosomal protein desmoglein 3 (Dsg3) induced by anti-Dsg3 antibodies, which are associated with pemphigus vulgaris (PV) (Jennings *et al.*, 2011). As shown in the top panels of Figure 2, PV IgG first binds Dsg3 on the surface of keratinocytes, consistent with its *in vivo* location in desmosomes. Over time, some Dsg3 moves to intracellular punctate structures, as shown at the 1- and 3-hour time points, suggesting that the autoantibody induces Dsg3 endocytosis. By utilizing fluorophores with different emission spectra, the authors were also able to follow the signal from desmoplakin, a second desmosomal protein. The center row in Figure 2 shows that desmoplakin remains mostly at the cell surface during Dsg3 endocytosis and rearranges into linear arrays prior to eventual degradation. The bottom row of Figure 2 shows merged images of the fluorescent antibody signals. As in the top row, green areas represent the anti-PV IgG signal, red areas the antidesmoplakin antibody signal, and yellow areas the overlay of the green plus red signals. Together, the images suggest a progressive loss of nondesmosomal Dsg3 from the plasma membrane via endocytosis, followed by internalization of desmosomal proteins and eventual degradation.

Patients with paraneoplastic pemphigus also express anti-Dsg3 antibodies. In a study by Saleh *et al.* (2012), phage display was used to isolate monoclonal antibodies against Dsg3 from a patient with paraneoplastic pemphigus. By incubating them with normal human skin and then detecting the immune complexes with a labeled secondary antibody, the authors showed that three of the antibodies generated the expected pattern in the basal epidermis (Figure 3a–c). A fourth autoantibody bound recombinant Dsg3 in an enzyme-linked immunosorbent assay, but did not stain the basal epidermis, indicating that recombinant proteins and fresh tissue may present altered epitopes (Figure 3d). As well, it was previously shown that anti-Dsg3 antibodies bind calcium-sensitive epitopes, so the authors reveal evidence that the staining here is also calcium-dependent in Figure 3f because it can be abrogated by preincubation of the skin with the

metal chelator EDTA. Figure 3e shows the staining pattern generated by incubating normal skin with the serum from a patient with PV as a positive control.

CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Answers and a PowerPoint slide presentation appropriate for journal club or other teaching exercises are available at http://dx.doi.org/10.1038/jid.2012.455.

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QUESTIONS

1. What is the purpose of Michel's solution?

A. Fix the tissue before detection.

B. Cross-link cellular components to retain the integrity of cellular structures.

C. Disrupt plasma membranes to give antibodies access to their target antigens.

D. Precipitate the immune complexes to preserve antigenicity.

2. How many additional antibodies are required to detect autoimmune complexes?

A. 1.

B. 2.

C. 3.

D. 4.

3. Which of the following techniques is more sensitive than immunofluorescence for the diagnosis of some autoimmune bullous diseases?

A. Light microscopy.

B. ELISA.

C. Dermatoscope.

D. Western blot.

Answers to the questions and an opportunity to comment on the article are available on our blog: http://blogs.nature.com/jid_jottings/.