Immuno-assays

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Introduction & AIMS

Pemphigus and pemphigoid are autoimmune bullous diseases that are characterized by autoantibodies to epithelial proteins. Immunoblot, immunoprecipitation and ELISA are laboratory techniques that can visualize to which epithelial protein(s) the autoantibodies are directed. ELISA furthermore can quantify the autoantibody titer. Furthermore we will discuss the keratinocyte binding assay and the keratinocyte footprint assay. In this chapter we will briefly outline how these techniques work and how results should be interpreted.

Learning Objectives

After reading this chapter you should be able to:

- Understand the principle of immuno-assays
- Interpret the results of immunoassays
- Decide if immuno-assays could be helpful in managing your patient
- Choose which immuno-assays to perform for individual patients

Immunoblotting

Immunoblotting is a qualitative test to identify which autoantigen(s) are involved a particular AIBD patient. Briefly, denatured skin proteins are separated and sorted on molecular mass by polyacrylamide gel electrophoresis (PAGE) and then transferred onto membrane filters to facilitate further incubation and washing steps. The filters are overlaid with patient serum and after washing bound IgG is stained. The antigens then become visible as purple bands and are identified by apparent molecular weight (Fig. 6.1) [1]. Skin proteins are obtained by extracting cultured human keratinocytes or human skin with the harsh soap sodium dodecyl sulphate (SDS). SDS

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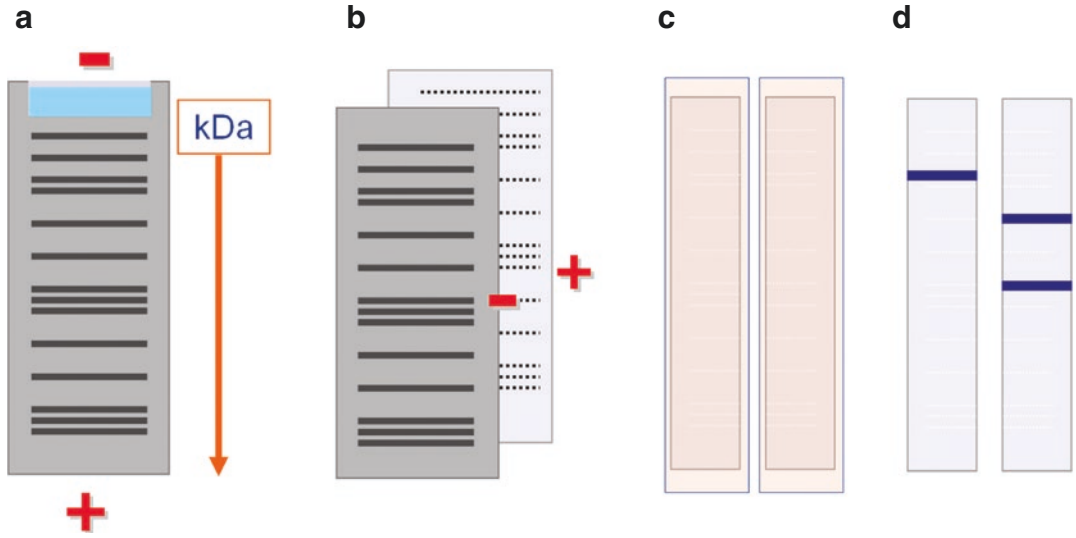

Fig. 6.1 Principle of immunoblotting. (**a**) Molecules in a skin protein extract are separated by gel electrophoresis. (**b**) The protein pattern is electrophoretically transferred to a membrane filter what facilitates further handling. (**c**) The filter is immersed in diluted patient serum. (**d**) Bound IgG is visualized by staining

has the ability to completely dissolve protein complexes including large structures as hemidesmosomes and desmosomes that contain major pemphigus and pemphigoid autoantigens. SDS is a negatively charged molecule and binds to proteins in an assumed SDS:protein ratio of 1.4. This destroys the native conformation of proteins that enroll and take on a linear shape. As all proteins become negatively charged with an even distribution of charge per unit mass they are fractionized according to size during electrophoresis.

Immunoblotting is a qualitative test to identify the targeted autoantigen.

SDS also has a disadvantage as it destroys conformational epitopes. An epitope is the part of the antigen that is recognized by the antibody and they have an average size of around 15 amino acids [2]. Epitopes are divided in two categories: linear epitopes that consist of a continuous stretch of amino acids and are thus determined by the primary structure and conformational epitopes formed by separate stretches of amino acids that lie close together in the native conformation of the protein and which are thus determined by the tertiary structure (Fig. 6.2). It is this last category of epitopes that is destroyed by the SDS and is missed in immunoblotting. For this reason immunoblotting is not suited for diagnosing pemphigus vulgaris (PV) or pemphigus foliaceus (PF) as the pathogenic epitopes of the autoantigens desmoglein 1 and 3 are largely conformational epitopes. In case of paraneoplastic pemphigus (PNP) however it is a good option as here immunoblotting has a reported 89% sensitivity and 100% specificity for detecting the simultaneous presence of antibodies to envoplakin and periplakin that is specific for PNP [3]. For identification of autoantibodies to pemphigoid antigens immunoblotting has a varying sensitivity and is not first choice when alternatives are available. For type VII collagen and BP230 ELISA's can now be commercially obtained. Diagnosis of anti-p200 pemphigoid seems to have high sensitivity but the quality of the dermal extract, which requires a sophisticated extraction procedure, is important and the assay is therefore only performed in a few highly specialized laboratories. Although plectin antibodies were found by immunoblotting in 4% of all pemphigoid patients the sensitivity is not known. Immunoblotting has additional value for detecting antibodies to BP180 which is the dominant antigen of the pemphigoid group. An ELISA for BP180 is available but can only detect antibodies to a small stretch of BP180 named NC16A that is

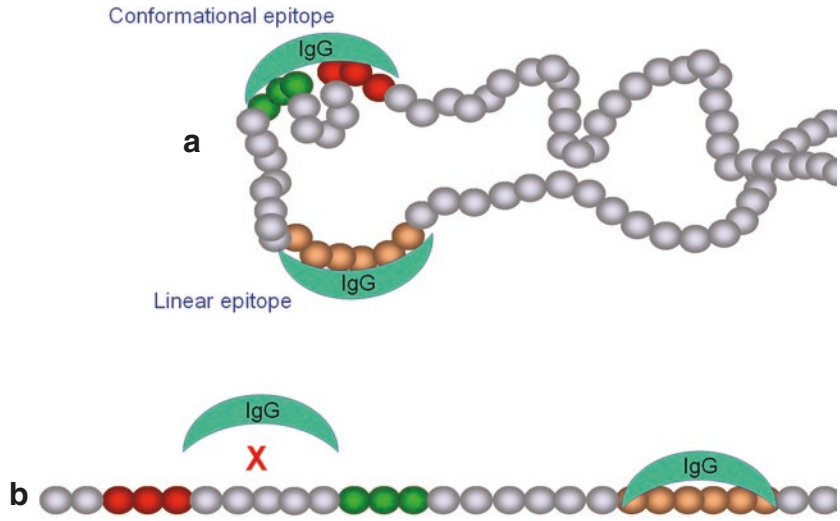

Fig. 6.2 Two classes of epitopes. (**a**) A native protein with a conformational epitope (red, green) is formed by two different parts of the molecule, while a linear epitope is formed by a continuous stretch of amino acids (orange). (**b**) A denatured protein looses its native conformation. Therefore IgG cannot bind anymore to the conformational epitope as it is destroyed while the linear epitope is still available

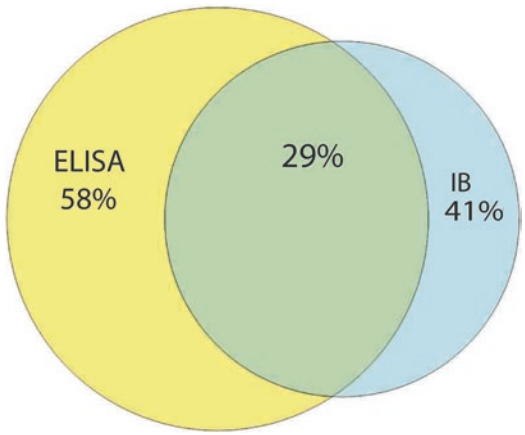

Fig 6.3 Venn diagram showing detection of anti-BP180 IgG antibodies in 357 patients with bullous or mucous membrane pemphigoid. When combined anti-BP180 IgG was found in 70% of the cases

reported to contain the major immunodominant epitopes. In contrast in immunoblotting the full length BP180 molecule is available. When ELISA and immunoblotting are compared immunoblotting detects 12% of the tested cases additional to ELISA (Fig. 6.3). Conversely immunoblotting misses half the cases found by ELISA indicating that also here loss of conformational epitopes plays a role.

Immunoprecipitation

Immunoprecipitation has played an important role in identification of the autoantigens involved in AIBD, the last one being alpha-2-macroglobulin-like-1 protein in PNP [4]. However as it is a labor intensive technique it is expensive and therefore not much used in routine diagnostics of AIBD.

The advantage of immunoprecipitation over immunoblotting is that the protein extracts for immunoprecipitation are prepared with soft soaps that do not denature proteins and the conformational epitopes therefore remain intact. Classical immunoprecipitation is performed with radioactive labeled proteins. Patient serum is first incubated with protein G-coupled beads. Protein G is a molecule that specifically binds IgG from the serum. After washing the beads are then added to the radioactive extract where the patient IgG will bind to the autoantigen(s) in question. The beads are then removed by centrifugation, washed and the IgG and the radioactive antigen(s) are eluted in SDS-PAGE sample buffer. The sample is then are separated and sorted on molecular size by polyacrylamide gel electrophoresis. Next the radioactive bands are visualized by fluorography and as in immunoblot indentified on basis of

molecular mass. Also unknown antigens can be identified by analyzing the radioactive band with advanced mass spectrometry methods. As working with radiochemicals is subject to strict regulations it is an easier option is to perform non-radioactive immunoprecipitation that is a combination of immunoprecipitation and immunoblotting. The procedure is largely the same but with non-radioactive substrates. After immunoprecipitation and gel electrophoresis the gel is blotted and the filter is incubated with a cocktail of antibodies that are specific for the antigens in question. After washing the blot can is stained to visualize which antigens have been precipitated from the extract (Fig. 6.4).

Immunoprecipitation has a higher sensitivity than immunoblot.

ELISA

Enzyme-linked immunosorbent assay (ELISA) is a technique that enables to measure the autoantibody response to a single autoantigen in a quantitative manner. ELISA's are commercially available and easy to perform, so can be introduced in every diagnostic lab. As all serological assays ELISA is based on the binding of patient IgG to the autoantigen. Principle of ELISA is that a small plastic well (200 µl volume) on a plastic plate is coated with a single antigen. The coated molecules are recombinantly produced and consists of the whole or particular part(s) of an antigen. The antigen is not denatured before coating and therefore contains both linear and conformational epitopes. Serum is brought into the coated well and if IgG to the antigen is present it will become bound. Next an anti-human IgG to which a special enzyme is conjugated is brought into the well. This will bind to the IgG and the more IgG is bound to the well the more of the enzyme will be bound. After washing the unbound IgG, a substrate is brought in the well that can be converted by the enzyme into a colored product. The more patient IgG is bound the more color will be produced and the amount of color is thus an indication of the amount of specific autoimmune IgG in the serum of the patient. This enables serological disease monitoring (Fig. 6.5). At January 2015 six different ELISA's were commercially available to respectively the pemphigus antigens desmoglein 1, desmoglein 3 and envoplakin, and to the pemphigoid antigens BP230, BP180 and type VII collagen. Of these the ELISA's to desmogleins 1 and 3 are most widely used as it enables discriminating between pemphigus vulgaris and pemphigus foliaceus. Pemphigus foliaceus has antibodies to desmoglein 1 but not to desmoglein 3, while mucosal dominant pemphigus vulgaris has IgG to desmoglein 3 only and mucocutane-

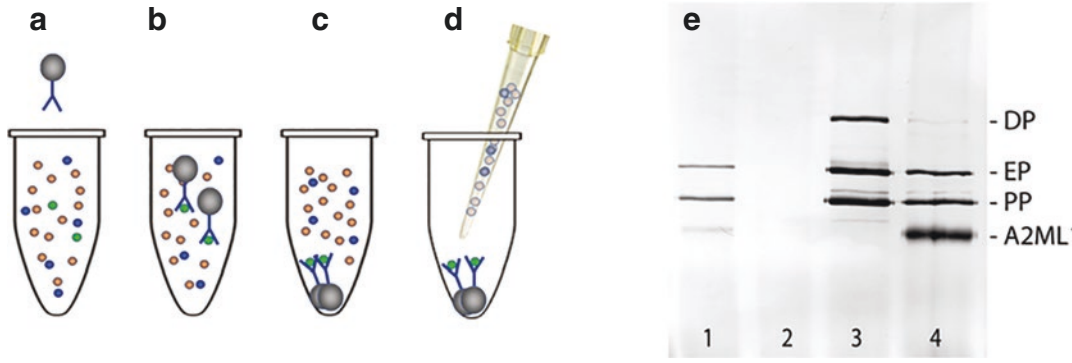

Fig. 6.4 Principle of non-radioactive immunoprecipitation. (**a**) Patient IgG bound to beads is added to a protein extract. (**b**) The IgG on the beads bind the disease causing antigen(s). (**c**) The beads are spun down by centrifugation. (**d**) The supernatant containing the other skin proteins is removed. (**e**) The antigen(s) are visualized by gel electrophoresis followed by immunoblotting. Here sera were analyzed for IgG to PNP antigens. Lane 1 PNP patient, lane 2 PV patient, lane 3 PNP patient, lane 4 PNP patient. *DP* desmoplakin, *EP* envoplakin, *PP* periplakin, *A2ML1* alpha-2-macroglobulin-like-2

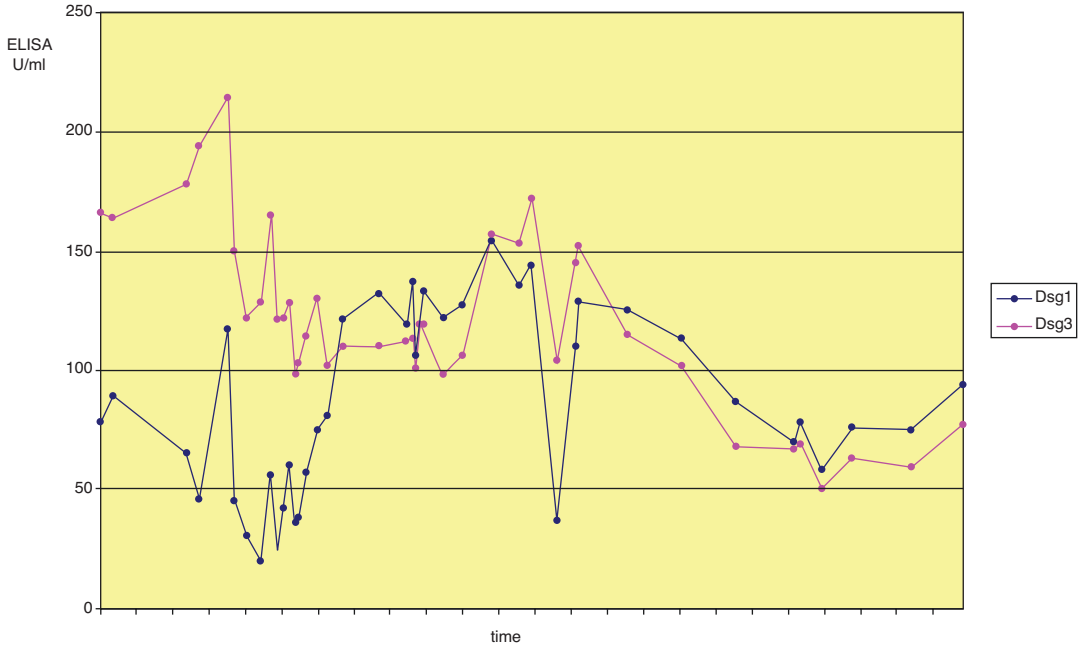

Fig. 6.5 Titer monitoring in pemphigus by ELISA to Dsg1 and 3. The patient was followed for eight years

ous pemphigus vulgaris to both desmogleins 1 and 3 [5]. The sensitivity to detect pemphigus is 89% by ELISA, which is slightly better than 86% by indirect immunofluorescence microscopy on monkey esophagus in our hands. Being quantitative these ELISA's are well suited to follow antibody titers (expressed as relative arbitrary units). The change in titer values from the desmoglein 1 ELISA fairly well corresponds to the activity of skin disease. However the results of the desmoglein 3 ELISA should be interpreted with more caution [6]. For about two-thirds of the patients there is a correlation with mucosal involvement but for the other third ELISA's may stay unchanged despite clinical improvement. Evidence is building that this is due to the presence of non-pathogenic antibodies to desmoglein 3 [7]. ELISA however cannot discriminate between pathogenic and non-pathogenic antibodies. Be aware that ELISA's may be false positive, desmoglein ELISA's up to 13% of tested samples and the NC16A ELISA for 11.3% [8, 9].

ELISA is a quantitative assay for monitoring disease activity.

The envoplakine ELISA has been developed to diagnose paraneoplastic pemphigus. Its sensitivity is estimated to be 63% and lower than immunoblotting [3]. The ELISA to type VII collagen was found to have 54% sensitivity due to approximately half of the patients having a very low undetectable serum titer [10]. For patients that have an ELISA detectable serum titer the type VII collagen ELISA values correspond well with disease activity. Above we already discussed the BP180 ELISA. This ELISA contains a small recombinant fragment NC16A, approximately 6.5% of the entire extracellular domain of BP180, but contains the immunodominant domains. Exact figures of its sensitivity are not know but based on the comparison with immunoblot results it can be estimated to be in the order of 70%. The BP230 ELISA is less sensitive and its diagnostic added value has to be found only 5% [11].

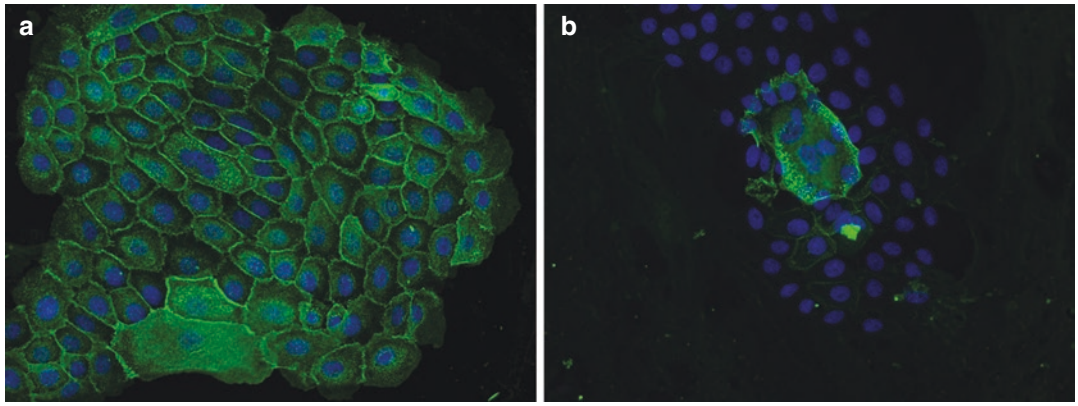

Fig. 6.6 Keratinocyte binding assay. (**a**) Pemphigus vulgaris IgG binds to all cells in a desmosomal pattern. (**b**) Pemphigus foliaceus IgG binds only to differentiated cells. IgG in green, nuclei in blue

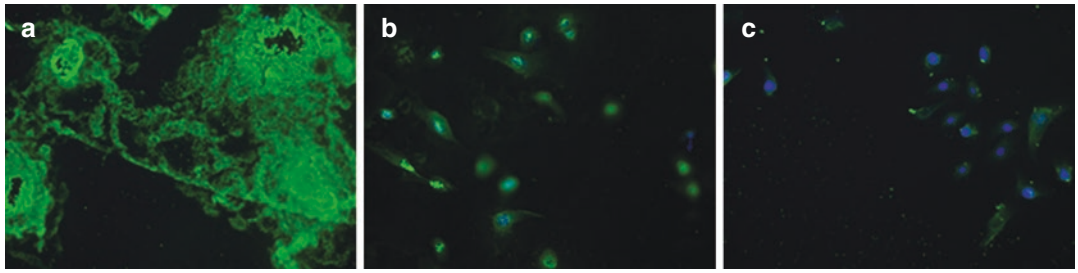

Fig. 6.7 Keratinocyte footprint assay. (**a**) IgG from anti-laminin-332 mucous membrane pemphigoid binds to the migration trails, but IgG from (**b**) epidermolysis bullosa aquisita and (**c**) anti-p200 pemphigoid do not. IgG in green, nuclei in blue

Keratinocyte Binding Assay

The Keratinocyte Binding Assay is the most sensitive and specific assay for pemphigus antibodies [12]. In this assay patient serum is added to keratinocytes cultured on high-calcium medium and incubated for 1 hour. If anti-desmosomal antibodies are present the IgG will bind in a desmosomal pattern. The assay is able to discriminate between pemphigus vulgaris and pemphigus foliaceus as all cells express desmoglein 3 but only differentiated cells express desmoglein 1 (Fig. 6.6). The sensitivity of the assay is 97%. The assay is useful to check for presence of antibodies if the desmoglein ELISA is negative, either because the titer is below the cut-off of the ELISA, or if antibodies to desmocollins are present, or if for some reason the IgG will not bind to the recombinant desmoglein on the ELISA plate, and to rule out false-positive ELISA's. It is not a quantitative assay thus not useful for monitoring antibody titers.

Keratinocyte binding assay is the most sensitive serum assay for pemphigus antibodies.

Keratinocyte Footprint Assay

The Keratinocyte Footprint Assay is a test that is specific for anti-laminin-332 antibodies [12]. Keratinocytes in culture need laminin-332 to attach to the surface of the culture dish. When migrating, the cells polarize and deposit precursor laminin-332 at the rear of the moving cell. This laminin-332 is left behind and forms a unique footprint trail. Keratinocytes are cultured on glass coverslips in low-calcium medium for three days after which coverslips are dried. The coverslips are then incubated with patient serum and bound IgG is visualized by immunofluorescence (Fig. 6.7). The

footprint trails do not contain other autoimmune bullous diseases antigens thus the specificity is 100%. Its sensitivity is estimated to be also almost 100%.

Keratinocyte footprint assay is a 100% specific for anti-laminin-332 mucous membrane pemphigoid.

Review Questions

1. Which assay is quantitative?
   a. Immunoblot
   b. ELISA
   c. Immunoprecipitation
   d. All three
2. The size of an epitope is on average
   a. 15 amino acids
   b. 50 amino acids
   c. 150 amino acids
3. ELISA values ……..parallel disease activity
   a. Always
   b. Most times
   c. Seldom
4. You have treated a patient with rituximab. What assay would you request to evaluate your therapy?
   a. Immunoblot
   b. ELISA
   c. Immunoprecipitation
   d. All three

Answers

1. b
2. a
3. b
4. b

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