Detection of Anti-basement Membrane Zone Antibodies in the Blister Fluid in Subepidermal Autoimmune Bullous Diseases

Ravindran Surya, Bobbili Tejasvi, Shrutakirthi D Shenoi, Sathish Pai, Chythra Rao, Raghavendra Rao

Abstract

Background: Subepidermal autoimmune bullous diseases (sAIBD) are diverse of conditions with clinicopathological overlap. Circulating autoantibodies in the serum can be demonstrated using indirect immunofluorescence (IIF) microscopy. Artificially split normal human skin is considered as an optimum substrate for the demonstration of anti-basement membrane zone (BMZ) antibodies using IIF in sAIBD; it not only helps to detect the presence of circulating antibodies in the serum but also helps to subclassify these conditions into “roof” and “floor” binding disorders. Aim: In this study, we evaluated the utility of IIF to detect anti-BMZ antibodies in the blister fluid of patients with sAIBD. Materials and Methods: Twenty-two patients with a clinical diagnosis of sAIBD were enrolled in the study. IIF of serum and blister fluid were done simultaneously using salt-split skin as a substrate. Results: Anti-BMZ antibodies could be detected in the blister fluid using IIF in all patients in the study group. Limitation: We could not do enzyme-linked immunosorbent assay of blister fluid. This would have given us the quantitative data of circulating antibodies in the blister fluid. Conclusion: Blister fluid offers an alternate source for the detection of autoantibodies in patients with sAIBD. It may be of particular help in children and in elderly with poor venous access.

Key words: Blister fluid, indirect immunofluorescence, subepidermal autoimmune bullous diseases

Introduction

Subepidermal autoimmune bullous diseases (sAIBD) are heterogeneous group of diseases characterized clinically by tense blisters on the skin with or without mucous membrane involvement. These include bullous pemphigoid (BP), epidermolysis bullosa acquisita (EBA), linear IgA disease (LAD), pemphigoid gestationis (PG), mucous membrane pemphigoid (MMP) and recently described anti-p200 pemphigoid.\(^\text{[1-3]}\) Autoantibodies in these conditions target the antigens present in the dermoepidermal junction triggering inflammation and blister formation.\(^\text{[4]}\) Histopathology in these conditions reveals subepidermal blisters with granulocytes-rich inflammatory infiltrates.\(^\text{[3]}\) The gold standard test to demonstrate the tissue-bound antibodies is direct immunofluorescence (DIF) microscopy of biopsy specimen obtained from the perilesional skin; it characteristically reveals linear staining IgG, IgA, and C3 classes of immunoreactants along the basement membrane zone (BMZ).\(^\text{[5]}\) Circulating antibodies in patient’s serum can be demonstrated by indirect immunofluorescence (IIF). Sensitivity of IIF microscopy depends on the substrate used; salt-split skin (SSS) is considered as the optimum substrate for IIF in patients with sAIBDs.\(^\text{[6,7]}\) This study was undertaken to identify the presence of anti-BMZ antibodies in the blister fluid of patients with sAIBDs using IIF microscopy and to compare it with that of the serum.

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How to cite this article: Surya R, Tejasvi B, Shenoi SD, Pai S, Rao C, Rao R. Detection of anti-basement membrane zone antibodies in the blister fluid in subepidermal autoimmune bullous diseases. Indian J Dermatol 2017;62:649-53.

Received: August, 2017. Accepted: November, 2017.
Materials and Methods

This study was conducted in a tertiary care teaching hospital over 1 year. This study was approved by the Institutional Ethical Committee Review Board. Twenty-two patients with a clinical diagnosis of subepidermal autoimmune blistering diseases (sAIBD) were enrolled in this prospective study. A perilesional biopsy was undertaken, and the clinical diagnosis was confirmed in each case by DIF microscopy. Tense, freshly erupted blister of 0.5 cm or more in size was chosen; using a 22-gauge needle, about 0.5 ml of blister fluid was aspirated and collected in an aliquot. Two milliliters of serum was collected simultaneously, and both serum and the blister fluid were stored in deep freezer until further use. SSS was prepared by overnight incubation of normal human skin (NHS) in 1 molar sodium chloride for 8 h in a rotator at 4°C in a refrigerator. This was then gently teased using a forceps to separate epidermis from the dermis. Specimen of SSS and NHS were then placed side by side and blocked together in optimum cutting temperature compound. Frozen sections of this block were obtained using a cryostat set at -26°C in a special glass slide with inbuilt adhesive properties (obtained from Hendley’s UK); frozen sections thus yielded both NHS and SSS sections side by side [Figures 1 and 2].

Serum and blister fluid were serially diluted (in 1:10 and 1:100 concentration) using phosphate buffer solution (PBS). This was then incubated with frozen sections (six sections per patient-three each for serum and blister fluid) of NHS/SSS for 60 min as shown in Figure 1. This was washed in PBS, and the secondary incubation was done for 60 min with fluorescein isothiocyanate conjugated IgG and IgA (DAKO, Denmark). The slides were again washed in PBS, mounted in buffered glycerol, and examined under fluorescence microscope. The intensity of staining was graded subjectively as strong positive (+++), moderately strong (++), weak (+), and negative (-); all the slides were read by the same investigator. Data were entered and analyzed using Statistical Package for the Social Sciences 15.0 (Chicago, SPSS Inc). The intensity of staining with IgG and IgA in blister fluid and serum was compared using Wilcoxon signed rank test; \( P < 0.05 \) was considered to be statistically significant.

Results

The mean age of patients in the study group was 57.5 years (Mean ± standard deviation 57.5 ± 13); sex distribution was equal among the study participants. Disease-wise distribution is described in Table 1. BP accounted for majority of the cases in this study \((n = 13)\), followed by EBA \((n = 7)\). Out of seven patients with EBA, six patients presented with inflammatory phenotype and were diagnosed initially as BP. This provisional diagnosis was revised to EBA after IIF of patient’s serum using salt-split study showed dermal staining pattern in these six patients.

Indirect immunofluorescence

In the present study, IIF on SSS detected the presence of anti-BMZ antibodies in all patients, both in the serum and blister fluid. However, IIF using NHS substrate was negative in five patients \((5/22)\) in the serum group and nine patients \((9/22)\) in blister fluid group making it an inferior substrate when compared to SSS, for the detection of anti-BMZ antibodies in patients with subepidermal autoimmune blistering diseases (sAIBDs).

Anti-BMZ antibodies belonged exclusively to IgG class in 19 patients \((12 \text{ patients with BP}, 6 \text{ patients with EBA, and } 1 \text{ patient with lichen planus pemphigoides})\). One patient with LAD showed BMZ staining exclusively with IgA class. A combined IgG and IgA class of autoantibodies were detected in two patients \((\text{one each of BP and EBA})\). All seven patients with EBA showed BMZ staining on the dermal side of the split (“floor” pattern), whereas rest of the patients exhibited staining on the
epidermal side of the split (“roof” pattern). There was no difference in the type of immunoreactants deposited and the pattern of staining between serum and blister fluid group [Figures 3 and 4].

In the BP subgroup [Table 2], intensity of staining with IgG class of antibodies was similar in both serum and blister fluid groups but for one patient in whom antibodies could not be detected in the blister fluid in 1:100 titer, that is, at higher dilution. On the other hand, in EBA group [Table 3], IIF was negative in blister fluid in 1:100 titer in two patients (both these patients showed weak staining in serum) and one patient showed less intense staining in 1:100 in blister fluid compared to serum (3+ vs. 2+). While other four EBA patients, as well as a case of lichen planus pemphigoides, showed no difference in the staining intensity between blister fluid and serum groups. A patient of LAD revealed moderately strong reaction with IgA in serum in contrast to blister fluid which showed weak reaction. Wilcoxon signed rank test indicated the asymptotic significance (two-tailed) of anti-IgG antibodies in 1:10 dilution between serum and blister fluid to be statistically significant ($Z = 2.236, P = 0.025$). No statistically significant association was noted with respect to anti-IgG antibodies in 1:100 dilution between serum and blister fluid ($Z = 1.394, P = 0.163$); and anti-IgA antibodies in 1:10 dilution between serum and blister fluid ($Z = 0.000, P = 1.000$).

**Discussion**

BP is the most common sAIBDs in clinical practice; it is characterized by tense blisters on urticated background with or without mucosal lesions. These clinical criteria, however, are not very specific to BP as these findings also can be seen in certain other sAIBDs, especially EBA. In the clinical setting, this distinction is important as BP has got a favorable prognosis in contrast to EBA which is known to run a protracted course often necessitating prolonged course of immunosuppressive therapy.[7] Given this scenario, we need a simple tool to distinguish these two conditions. Gammon et al. in 1984 described IIF performed on SSS as an ideal test to differentiate anti-lamina lucida antibodies (e.g., BP) from anti-sublamina densa antibodies (e.g., EBA).[8] They incubated the NHS in 1 molar sodium chloride for 72 h at 4° centigrade; subsequently, the epidermis was dislodged from the dermis by applying lateral traction. We tried a modification of the original technique; we could obtain the dermoepidermal separation by overnight incubation of NHS in 1 molar sodium chloride. In this study, we blocked NHS and SSS side by side; frozen section thus taken from this block contained both NHS and SSS. This is the deviation from our previous practice of taking separate sections of NHS and SSS in two different glass slides. Both these techniques

**Table 1: Disease-wise distribution and mean age at presentation**

| Disease                | Total number of patients | Mean age at presentation |
|------------------------|--------------------------|--------------------------|
| Bullous pemphigoid     | 13                       | 65.2                     |
| Epidermolysis bullosa acquisita | 7               | 49.5                     |
| Linear IgA disease     | 1                        | 10                       |
| Lichen planus pemphigoides | 1             | 51                       |

**Table 2: Intensity of staining with immunoglobulin G on salt-split skin with immunoglobulin G in bullous pemphigoid**

| Intensity of staining | Serum (1:10/1:100) | Blister fluid (1:10/1:100) |
|-----------------------|--------------------|-----------------------------|
| Strong (+++)          | 4/1                | 4/1                         |
| Moderately strong (+) | 4/5                | 4/5                         |
| Weak (+)              | 5/7                | 5/6                         |
| Negative              | Nil                | Nil/1                       |

**Table 3: Intensity of staining with immunoglobulin G on salt-split skin with immunoglobulin G in epidermolysis bullosa acquisita**

| Intensity of staining | Serum (1:10/1:100) | Blister fluid (1:10/1:100) |
|-----------------------|--------------------|-----------------------------|
| Strong (+++)          | 3/3                | 2/1                         |
| Moderately strong (+) | 3/nil              | 2/2                         |
| Weak (+)              | 1/4                | 3/2                         |
| Negative              | Nil                | Nil/2                       |

**Figure 3:** Linear staining of IgG on the epidermal side of the split in blister fluid (a) and in the serum (b) in a patient with bullous pemphigoid (red circle represents the level of split) fluorescein isothiocyanate X200

**Figure 4:** Linear staining of IgG on the dermal side of the split in blister fluid (a) and in the serum (b) in a patient with epidermolysis bullosa acquisita (red circle represents the level of split) fluorescein isothiocyanate X200
have helped us to reduce the time required to perform IIF using two substrates.

The previous studies have shown that blister fluid in BP contains various inflammatory mediators, including interleukin-1 (IL-1), IL-2, IL-4, IL-6, IL-8, IL-10, tumor necrosis factor-α, and interferon-γ. [4-13] Wakugawa et al. have shown elevated levels of eotaxin and IL-5 in blister fluid of BP patients. The eotaxin levels in blister fluid significantly correlated with the number of eosinophils in the dermis. [12] In fact, levels of cytokines were higher in blister fluid when compared to the serum; this could be related to the localized production of these cytokines by the keratinocytes during the inflammatory process. [11] However, only a few studies have investigated the presence of antibodies in blister fluid. [4,13,14] In 1970, Bean detected BMZ antibodies in the serum and blister fluid of three BP patients. [13] Zhou et al. studied IIF of serum and blister fluid in 35 patients with sAIBDs. This included BP (30 patients), PG (2), LAD (2), and one patient with MMP. Anti-BMZ antibodies were detected in the SSS in 24 patients (twenty-one cases of BP, two cases with LAD, and one patient with MMP) by IIF in their series. The antibody titer in blister fluid has been found to be the same (16 patients) or one dilution less (five patients) than that in serum in BP. They concluded that, unlike cytokines, antibodies (mainly IgG1 and IgG4) in the blister fluid are derived from the vascular compartment through the process of diffusion. [4] Daneshpazhooh et al., in their series of 35 patients with sAIBDs detected anti-BMZ antibodies in the blister fluid in 28 patients, using IIF. Interestingly, in one of their BP case, IIF was positive in blister fluid but was negative in serum; this patient had limited disease on the scalp. The authors concluded that in patients who have recently been affected by BP or suffer from limited BP, IIF on blister fluid might be more useful than that on serum. They also showed that the use of both serum and blister fluid samples in BP patients increased the sensitivity of IIF in detecting IgG anti-BMZ antibodies from 92% to 96%. [14] Patsatsi et al. performed BP enzyme-linked immunosorbent assay (ELISA) (BP 180 and 230) of blister fluid in thirteen newly diagnosed BP patients, before starting treatment. [15] Antibodies could be detected by ELISA in nine patients (69.2%) in blister fluid in contrast to serum where it could be found in eleven patients (84.6%). Sensitivity of IIF in this group of patients with BP was 61.5% (positive in 8 of 13 patients). They concluded that the sensitivity of detecting anti-BMZ antibodies in blister fluid using traditional diagnostic techniques such as IIF was comparable with that of the ELISA technique.

We could detect anti-BMZ antibodies in the blister fluid in all patients in the study group. Autoantibodies belonged predominantly to IgG class; anti-IgA antibodies were detected only in three cases. The previous studies have reported autoantibodies of IgA class in 35%-40% patients with BP; [4,14] in contrast, it was detected in only one patient of BP in the present study. It is not clear yet whether the presence of antibodies of IgA class has any effect on the clinical course and/or response to treatment in BP patients. The cohort in the present study included two children (one each of LAD and EBA); IIF using blister fluid was positive on SSS in both these patients.

Conclusion

Blister fluid as an alternative source for the detection of anti-BMZ antibodies using IIF. It is less traumatic than venipuncture and is particularly useful in children and elderly, from whom it may often be difficult to obtain the blood samples. The aspiration of blister fluid is also therapeutic and is often used to relieve discomfort of multiple tense fluid-filled lesions and can also provide large amount of materials for the diagnosis and investigations.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

What is new?

- Blister fluid in sAIBD contains autoantibodies and other cytokines and may be used instead of serum for IIF microscopy
- This may be particularly useful technique in patients with poor venous access and children.

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