Identification of a Basement Membrane Zone Antigen Reactive with Circulating IgA Antibody in Ocular Cicatrical Pemphigoid

Eileen Pazderka Smith,* † Ted B. Taylor, † Laurence J. Meyer,* † and John J. Zone* †

*Dermatology Section, Department of Internal Medicine, Veterans Affairs Medical Center, Salt Lake City; and †Division of Dermatology, Department of Internal Medicine, University of Utah School of Medicine; Salt Lake City, Utah, U.S.A.

Ocular cicatrical pemphigoid is a rare vesiculobullous disease characterized by linear deposition of IgG and/or IgA along the basement membrane zone of conjunctival biopsies. This study identifies a tissue antigen detected by ocular cicatrical pemphigoid patient sera.

Patient selection was based on the presence of only ocular involvement and a positive direct immunofluorescence of conjunctiva. We evaluated patient and control sera using indirect immunofluorescence of basement membrane zone separated skin, Western blot, and purified antibodies from nitrocellulose and epidermal sheets.

Direct immunofluorescence performed on the patients' conjunctival biopsy showed linear deposition of IgA along the basement membrane zone in all seven patients, and five of seven also demonstrated deposition of IgG along the basement membrane zone. Indirect immunofluorescence performed on the patients' sera demonstrated linear deposition of IgA along the epidermal side of the basement membrane zone of ethylenediaminetetraacetic acid–separated skin in all seven patients. IgA titers ranged from 1:20 to 1:80. No IgG was detected. Immunoblots detected IgA binding to a 45-kD antigen in all patients as well as sporadic IgA binding to a number of other proteins. Immunoblots stained with sera from patients did not show reactivity to the 230- or 180-kD bullous pemphigoid antigens or the 97-kD linear IgA bullous dermatosis antigen. Eluting IgA from the 45-kD region and other regions revealed that only antibodies eluted from the 45-kilodalton region bound linearly to the basement membrane on separated skin. Purification of IgA using epidermal sheets confirmed that the antibody responsible for staining on indirect immunofluorescence bound to the 45-kD region on Western blot. Sera from normals and patients with bullous pemphigoid, dermatitis herpetiformis, and linear IgA bullous dermatosis failed to demonstrate basement membrane zone IgA on elution of the 45-kD region.

We conclude that these ocular cicatrical pemphigoid sera contain a unique IgA antibody that binds to a 45-kD basement membrane zone antigen. Key words: bullous disease/autoimmune/Western Blot/purification. J Invest Dermatol 101:619–623, 1993

Cicatrical pemphigoid (CP) is a chronic subepidermal, autoimmune, vesiculobullous disease that primarily affects mucosal surfaces [1–3]. Linear deposition of IgG and/or IgA along the basement membrane zone (BMZ) is characteristic of CP, but very little information is available concerning the antigen responsible for antibody binding in CP.

Traditionally, serum of patients with CP has rarely been shown to demonstrate the presence of circulating antibodies that bind to the BMZ using indirect immunofluorescence (IIF) techniques [4–11]. These studies were done with a variety of substrates and predominantly searched for IgG-class antibodies.

Antigenic proteins extracted from the skin have been demonstrated in bullous pemphigoid (BP) [12,13], epidermolysis bullosa acquisita [14], pemphigus vulgaris [15], pemphigus foliaceus [16], and herpes gestationis [17], but the antigen responsible for antibody binding in CP is still controversial. Direct immunoelectron microscopy studies of patients with CP reveal localization of the immunoreactants within the lamina lucida of the BMZ [18,19]. Despite localization of immunoreactants within the lamina lucida, studies have shown that the antigen involved in CP may be distinct from the BP antigen [18,20–22]. On the other hand, Bernardi et al. and Niimi et al. reported identification of a CP antigen that is similar to the bullous pemphigoid antigen [23,24].

In the present study we enrolled seven patients with clinical evidence of ocular cicatrical pemphigoid (OCP). In all seven patient sera we identified IgA antibodies using BMZ-separated skin and we identified a 45-kD BMZ antigen that binds IgA antibodies. The antigen was identified in epidermal extracts using Western blots. BMZ specificity was confirmed using immunoaffinity-purified antibodies.

MATERIALS AND METHODS

Patient Selection Seven patients meeting the following criteria were enrolled in our study: clinical evidence of ocular disease (excluding those with oral and skin disease) and a positive conjunctival biopsy for IgG and/or IgA. Patients with a negative direct immunofluorescence (DIF) were excluded from the study.

Sera Seven OCP sera meeting the above criteria were evaluated. Controls included five BP, four dermatitis herpetiformis, two linear IgA bullous dermatosis patients, and eight normal controls free from bullous disease.

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Separated Skin  Skin separated at the BMZ has been shown to be a good substrate for IIF testing [18, 25–28]. Various methods have been developed for separation of epidermis from the dermis using sodium chloride or ethylenediaminetetraacetic acid (EDTA) [18, 26–28]. EDTA-separated skin was used in our IIF techniques and was prepared as previously described by Meyer et al [27].

Immunofluorescence  DIF testing was performed on all conjunctival biopsies following standard techniques [27–29]. IIF testing was performed on all sera for IgG and IgA (Cappel Laboratories, Malvern, PA) following the standard IIF technique [27–29].

Epidermal Extracts  Epidermis used for polyacrylamide gel electrophoresis was separated from dermis at the BMZ by heating at 56°C and extracts were made from the epidermis as described previously [30]. The epidermal extract was stored at −70°C. There was no loss or change in antigenicity with storage.

Electrophoresis and Western Blots  Polyacrylamide gel electrophoresis was performed using epidermal extracts by the method of Laemmli [31], with use of a 9.5% acrylamide running gel and 5% acrylamide stacking gel. Following polyacrylamide gel electrophoresis, proteins were transferred electrophoretically onto nitrocellulose, as described by Towbin et al [32]. Strips of nitrocellulose were incubated with all sera at a 1:10 dilution, then with biotinylated goat anti-human IgA, and were finally incubated in an avidin-biotin-peroxidase complex. Protein bands were stained with 4-chloro- naphtol and hydrogen peroxide (H2O2) [33].

Western Blot Immunofinity  Protein bands on Western blots from Laemmli gels were used as immunofinity substrates for purification of antibodies directed against specific proteins in the epidermal extract as described by Olmsted [34] and modified by Smith and Fisher [35]. Briefly, vertical strips were cut from the end of nitrocellulose containing the antigen in question and incubated with sera containing the antigen-specific antibody. A second incubation with goat anti-human IgA and, finally, an avidin-biotin-peroxidase complex, was incubated with the antigen-specific antibody. The protein bands were stained with 4-chloro-naphtol and H2O2 for identification of the bands of interest [33]. Horizontal strips of nitrocellulose containing the antigen of interest were cut using the end strips as a template and incubated with a 1:5 dilution of sera for 2 h. The antibody was eluted from the horizontal strips using 20 mM sodium citrate, pH 3.2. The eluent was then neutralized with 2 M tris buffer, pH 8, and stored at 4°C until used in our Western blot and IIF techniques. A slight modification of the IF technique was used to detect eluted antibodies. Incubation time was increased to 4 h to assure binding of the eluted antibody and increased sensitivity.

Basement Membrane Zone Immunofinity  EDTA-separated skin was used as an immunofinity substrate for purification of anti-BMZ antibodies as described previously [28]. Briefly, abdominoplaty skin was incubated overnight at 4°C in 20 mM EDTA. The epidermis was removed from the dermis and placed in a humidifying chamber with the BMZ up. A 1:7 dilution of serum was incubated with the epidermal sheet for 2 h at room temperature. The antibodies were then eluted from the epidermal sheet with 20 mM sodium citrate, pH 3.2. The eluates were neutralized with 2 M tris buffer, pH 7.5 and stored at 4°C until used for IIF and Western blot techniques.

RESULTS

Immunofluorescence Studies  DIF performed on the patients' conjunctival biopsy showed linear deposition of IgA along the BMZ in all seven patients, and five of seven also demonstrated deposition of IgG along the BMZ. IIF performed on the patients' sera demonstrated linear deposition of IgA along the epidermal side of the BMZ of EDTA-separated skin in all seven patients (Fig 1). There was no dermal binding of antibody. IgA titers ranged from 1:20 to 1:80. There was no detectable IgG. Normal control sera failed to demonstrate basement membrane zone antibody.

Immunoblot Epidermal Extracts  Immunoblots stained with these low-titer OCP sera demonstrated binding of IgA to numerous proteins (Fig 2). It was not apparent from Western blot alone which, if any, of the antibodies bound on Western blot were responsible for the BMZ binding seen on IIF. To determine which band was responsible for the linear staining of the BMZ seen on the IIF Western blot, immunoaffinity purification was performed. Initially each protein band seen on Western blot from the serum of patient 2 (Fig 2, lane 2) was eluted from nitrocellulose. The only IgA antibody to bind to the BMZ in a linear fashion was from a band in the 45-kD region (Fig 3). Immunoaffinity-purified antibodies from the other regions of the immunoblot from patient 2 did not bind to the BMZ. Subsequently antibody from the 45-kD region was eluted from all OCP patients' sera and BMZ reactivity was confirmed in all sera. It was not feasible to elute all bands from all patients, but basement membrane zone immunofinity purification was used to further verify the importance of the 45-kD band.

Immunoblots stained with OCP sera using epidermal extracts containing the 230-kD, 180-kD BP antigens (Fig 4) or the 97-kD antigen (not shown) did not show reactivity at those molecular weight regions. (See Table I). Immunoblots stained with control sera using epidermal extracts and serum diluted 1:10 showed nonspecific reactivity with IgA class antibodies in the 45-kD region. This included each of the five BP, four dermatitis herpetiformis, two linear IgA bullous dermatosis sera, and two of the eight normal sera (not shown). However, when antibodies from control sera that bound to this region were eluted from nitrocellulose and used in our IIF procedure they did not bind back to the BMZ. Immunoblots stained with sera used at low dilutions (1:10) frequently produce

Figure 1. Indirect immunofluorescence. IIF of OCP sera showing linear deposition of IgA along the epidermal side of the BMZ. Skin separated at the BMZ with 20 mM EDTA was used as substrate.

Figure 2. Immunoblot. Western blot using epidermal extract rich in 45-kD antigen and incubated with OCP and normal sera. Lanes 1–7, OCP sera; lanes 8 and 9, normal control sera; and lane 10, no primary control, which is not incubated with serum. Nitrocellulose was incubated with biotinylated goat anti-human IgA.
IgA binding in the 40–60-kD region. When many of these antibodies are eluted, binding to various antigens within the epidermis occurs. We believe this to represent non-specific keratin binding. In contrast, when the IgA antibodies from OCP sera were eluted from nitrocellulose, they specifically bound to the BMZ of separated skin in a linear fashion and the eluted antibody also bound back to the 45-kD region on immunoblot.

**Basement Membrane Zone Immunoaffinity** Immunoblots (as described above) were used as immunoaffinity substrates to determine whether the antigen detected on immunoblot corresponded with the antigen detected by indirect immunofluorescence. To further support the idea that the circulating IgA in OCP sera binds to this newly described 45-kD BMZ antigen, we used epidermal sheets to purify the BMZ-binding IgA antibodies. These purified antibodies were then compared to whole sera of the same patients. In all cases there was binding of the BMZ-purified antibody to the epidermal side of skin that had 45-kD region (Fig 5), demonstrating that the antibody eluted from the BMZ binds to an epidermal antigen in the 45-kD region.

The data produced using the various techniques on all OCP sera are summarized in Tables I and II.

**DISCUSSION**

CP is a rare vesiculobullous disease resulting in separation of the basement membrane between the epithelium and underlying stroma [1–3]. Because immune deposits of IgA and IgG as well as complement have been shown to be deposited at the BMZ by DIF and some patients have circulating antibodies in their serum that bind to the BMZ, it is likely that immunologic mechanisms are involved in the pathogenesis of the disease.

In this study, serum from seven patients with OCP contained IgA antibodies that bound to the BMZ of normal human skin, as demonstrated by IIF. Our seven OCP patients had disease limited to their eyes and BMZ IgA autoantibodies in their serum. The IgA autoantibodies all bound to the epidermal side of skin that had

**Table I. Summary of Indirect Immunofluorescence and Western Blot Analyses of OCP Patient Sera**

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Serum Titer, Separated Skin*</th>
<th>Western Blot (45-kD Antigen)</th>
<th>Western Blot (97-, 180- and 230-kD Antigen)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:80 Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>2</td>
<td>1:80 Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>3</td>
<td>1:80 Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
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<td>Negative</td>
</tr>
<tr>
<td>7</td>
<td>1:20 Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
</tbody>
</table>

* Evaluation of serum IgA-class basement membrane antibody titer by IIF using 20 mM EDTA-separated skin as substrate. Reported as last titer showing staining of the epidermal side of 20 mM EDTA-separated skin.

* Western blot of epidermal extracts rich in BMZ antigens incubated with patient serum diluted 1:10 and biotinylated goat anti-human IgA or IgG.

![Figure 3. Indirect immunofluorescence. IIF of OCP immunoaffinity-purified antibody showing linear deposition of IgA along the BMZ on the epidermal side. Skin separated at the DMZ with 20 mM EDTA was used as substrate.](image3)

![Figure 4. Immunoblot. Western blot using epidermal extract rich in 180- and 230-kD BP antigens were incubated with OCP and normal sera. Lanes 1 and 2, BP sera known to contain the 180- and 230-kD antigens, respectively. Lanes 3–7, OCP sera; lanes 8 and 9, normal control sera. BP control strips were incubated with biotinylated goat anti-human IgG and OCP and normal control strips were incubated with biotinylated goat anti-human IgA.](image4)

![Figure 5. Immunoblot. Western blot using epidermal extract rich in the 45-kD antigen were incubated with OCP sera and BMZ-purified antibodies. Lane 1, molecular weight standards; lane 2, OCP sera; lane 3, BMZ-purified antibody from the serum shown in lane 2; lane 4, no primary control, which is not incubated with serum. Nitrocellulose was incubated with biotinylated goat anti-human IgA.](image5)
purification of the IgA antigen-specific antibody from OCP sera. These purified antibodies were used to probe epidermal extracts and BMZ-separated skin for reactivity with the 45-kD antigen. ND, not done.

Several studies have shown that sera from CP patients have circulating IgG and/or IgA antibodies that bind to the BMZ by IIF [4–11]. These studies were performed using a variety of substrates and predominantly searched for IgG-class antibodies. Perhaps our success in identification of IgA-class circulating antibodies may be due to the patient population selected and the substrate used for IIF. Our patient population was limited to CP patients with only eye disease. The other studies included CP patients with buccal mucosal involvement and many even had cutaneous involvement. Many of the studies did not look for or were unable to find IgA-class circulating antibodies. Identification of circulating antibodies has been difficult in the past but it has been shown that skin separated at the BMZ is a good substrate for IIF testing [18,25–28]. In a recent cooperative study, Sarret et al. [36] evaluated sera of 11 patients with CP, which demonstrated positive IIF using normal human skin separated at the BMZ and that circulating IgA antibody was the single immunoglobulin class in 55% of the sera. Our study has confirmed that IgA is the predominant immunoglobulin class present in OCP serum and that BMZ split skin is a good substrate for demonstrating the presence of circulating antibodies. Further support for the potential role of IgA includes: 1) the fact that IgA is the predominant mucosal immunoglobulin and is likely to be important in the pathogenesis of mucosal immune disorders such as CP, and 2) dapsone, which has been shown to be effective in treating IgA dermatoses, has also been shown to be effective in the treatment of CP [37].

For many years, there has been controversy regarding the nature of the antigen(s) that react with autoantibodies in CP. Initial reports of the isolation of antigens with molecular weights ranging from 90 to 280 kD that predominantly bound IgG have not been confirmed [21–24]. Two reports from Bernard et al. and Niimi et al. have described IgG antibodies reactive with antigens similar to the 230-kD and 180-kD of BP [23,24]. We describe a 45-kD BMZ antigen that binds circulating IgA from our select group of CP patients. What role this antigen plays in the pathogenesis of CP is yet to be determined. We believe that this antigen may be a component of a larger molecular weight protein and identification of this larger molecular weight protein may be impossible by Western blot. The methodology used in immunoblotting is substantially different from that employed in immunoprecipitation; the substrate is epidermal sheets from surgical procedures, and the tissue is homogenized directly into sodium dodecyl sulfate (SDS) and reduced. The denaturing effect of the SDS renders this technique less sensitive than immunoprecipitation, and reduction may produce antigenically inactive proteins. Our future goal will be to perform immunoprecipitation with these sera to determine whether the 45-kD protein is actually a component of a larger molecular weight protein.

**REFERENCES**


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**Table II.** Summary of Immunoaffinity-Purified Antibodies

<table>
<thead>
<tr>
<th>Nitrocellulose- Eluted Antibody</th>
<th>Epidermal Sheet- Eluted Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Separated Skin</td>
</tr>
<tr>
<td>1</td>
<td>Positive</td>
</tr>
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<td>2</td>
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</tr>
<tr>
<td>7</td>
<td>Positive</td>
</tr>
</tbody>
</table>

* Nitrocellulose and epidermal sheets were used as immunofluorescence substrates for purification of the IgA-antigen-specific antibody from OCP sera. These purified antibodies were used to probe epidermal extracts and BMZ-separated skin for reactivity with the 45-kD antigen. ND, not done.
*Arch Dermatol* 128:54–57, 1992