Detection of Pemphigus Vulgaris and Pemphigus Foliaceus Antigens by Immunoblot Analysis Using Different Antigen Sources

Takashi Hashimoto, M.D., Marilia M. Ogawa, M.D., Akira Konohana, M.D., and Takeji Nishikawa, M.D.
Department of Dermatology, Keio University School of Medicine, Shinjuku, Tokyo, Japan

In an immunoblot analysis with human epidermal extract as a source of antigens, all (28/28) pemphigus vulgaris (Pv) sera showed a specific reactivity with a 130-kD protein. Several, but not all, Pv sera reacted with similar antigens in both a bovine muzzle desmosome preparation and extract of cultured human squamous carcinoma cells. On the other hand, some pemphigus foliaceus (Pf) sera exhibited reactivity with a 150-kD protein, which is most likely desmoglein I, in both the human epidermal extract and the bovine desmosome preparation, but no Pf serum reacted with this antigen in the squamous carcinoma cell extract. Furthermore, 4/16 Pv sera also reacted with a 150-kD protein in the desmosome preparation, which seemed to be the same as Pf antigen. These results show a relationship between antigens of both Pf and Pv and desmosomes, as well as heterogeneities of both Pv and Pf antigens in terms of antigenic molecules or epitopes. Furthermore, this study presents the possibility that immunoblot analysis can be routinely used for differentiation of Pv and Pf antibodies. J Invest Dermatol 94:327–331, 1990

In a number of reports attempts have been made to identify the antigens reactive with the anti-intercellular (IC) antibodies from pemphigus patients [1–6]. Stanley, Yaar, Hawley-Nelson, and Katz have identified pemphigus antigens by means of immunoprecipitation using cultured murine and human epidermal cells metabolically radiolabeled with sugars and amino acids [7]. The pemphigus vulgaris (Pv) antigen was identified as a dimer of a 130-kD glycoprotein and an 80-kD protein linked by a disulfide bond. In contrast, pemphigus foliaceus (Pf) sera studied by the same method did not demonstrate any protein band. However, on immunoblot analysis of extracts from human epidermis or human cultured epidermal cells, about half of the Pf sera specifically recognized a 160-kD band. A series of the studies in their laboratory further supported these results [8–10]. Later, other studies indicated that the 160-kD protein recognized by Pf sera was identical to desmoglein I, a transmembranous constituent of desmosome [11–13].

Recently, Eyre and Stanley [14] have shown that the Pf antigen possessed two different epitopes: one was conformationally calcium-sensitive and the other was resistant to chelation of calcium and to sodium dodecyl sulfate (SDS) treatment. All Pf sera precipitated an antigen complex of 260-kD, 160-kD, 110-kD, and 88-kD polypeptides from an iodinated human epidermal sample extracted under mild conditions in the presence of calcium. Furthermore, using the same technique, they revealed that Pv sera precipitated a complex of 210-kD, 130-kD, and 85-kD polypeptides. Because the 85–88kD polypeptides recognized by both Pf and Pv sera were identical and the 130-kD polypeptide in Pb antigen complex and the 160-kD polypeptide in Pf complex showed similar characteristics, they suggested the close relationship between Pb and Pf antigens [15]. In all reports by Stanley and his colleagues [7–15], they insisted that their immunoblot system did not demonstrate Pb antigens.

Conversely, Jones, Yokoo, and Goldman [16] reported that immunoblot analysis using desmosome preparation from bovine tongue as a source of antigens revealed a 140-kD protein specifically recognized by some of Pf sera, in addition to a 160-kD protein recognized by Pf sera. They could not detect the 140-kD Pf antigen when desmosome preparation from bovine muzzel epithelium was used for immunoblotting. Furthermore, other authors reported considerably different antigens for Pb by immunoblotting using a variety of antigen sources [17–19].

Thus, the pemphigus antigens do not seem to be fully characterized yet. We report here the results of our immunoblot analyses that enabled the demonstration of both the Pb and Pf antigens from three different sources of antigens.

MATERIALS AND METHODS

Sera We selected 28 Pb, 13 Pf, and 10 pemphigus erythematosus patients by typical clinical, pathologic, and immunopathologic criteria. Because the latter two types of pemphigus showed identical results in this study, we put both together and refer to them as Pf. All sera obtained from our patients showed anti-IC antibodies at titers of 1:40 to 1:2560 by indirect immunofluorescence (IIF) with nor-
nal human skin as a substrate, and were stored at $-20^\circ$C. Sera from 15 normal volunteers were used as control sera.

**Epidermal Extraction** Skin pieces of adult foreskin were incubated with 1000 U dispase (Goudou-Shusei, Tokyo, Japan) in Dulbecco’s modified Eagle’s medium (DMEM: Gibco Laboratories, Grand Island, NY) at 37°C for 45 min [20]. Epidermis was separated and extracted according to the method by Labib, Anhalt, Patel, Mutasim, and Diaz [21] with several modifications. Briefly, epidermis was homogenized on ice with 3 ml of 1.5% SDS-0.0625 M Tris-HCl buffer, pH 6.8, supplemented with 5% 2-mercaptoethanol (2-ME), 2 mM phenylmethylsulfonyl fluoride (PMSF: Sigma Chemical Co., St. Louis, MO), and 10 μg/ml of pepstatin A, antipain, leupeptin, and chymostatin (Sigma Chemical Co.), boiled for 5 min, and centrifuged at 15,000 × g for 30 min. Supernatant was harvested and stored at $-70^\circ$C as aliquots.

**Desmosome Preparation** Desmosome was prepared from bovine muzzle epidermis by the method described by Skerrow and Motulsky [22,23]. (This work was performed by Dr. Konohana during his stay with the Department of Medicine [Dermatology], University of Wales College of Medicine, Cardiff, U.K.). Before being applied on SDS-polyacrylamide gel electrophoresis (PAGE), the preparation was boiled for 2 min in sample buffer (2% SDS-0.0625 M Tris-HCl buffer, pH 6.8, containing 5% 2-ME).

**Cell Culture** KU8 cells, a well-characterized squamous carcinoma cell line derived from a penile carcinoma, was a kind gift from Dr. H. Tazaki, Department of Urology of Keio University [24]. KU8 cells were maintained in DMEM supplemented with 10% fetal calf serum (Gibco Laboratories). To determine the presence of pemphigus antigen, we stained living cells by IIF, according to the method described by Stanley et al. [7]. For immunoblotting analysis, cells were washed with phosphate-buffered saline (pH 7.4) and scraped with a rubber policeman into the same buffer used for the extraction of epidermis. The following immunoblotting procedure was exactly the same for the extraction from epidermis and that from KU8 cells.

**Immunoblotting** SDS-PAGE was performed by Laemmli’s method [25], with a 6% separating gel. Separated proteins were electrophoretically transferred to a nitrocellulose sheet (Schleicher and Schnell, Dassel, West Germany) according to the method described by Towbin, Staehelin, and Gordon [26]. Strips of the blotted sheet were first blocked with 3% powdered skim milk in Tris-HCl buffered saline (TBS, pH 8.0) for 1 h at room temperature, treated overnight at 4°C with patients’ or normal sera diluted at 1:40 with 3% milk powder in TBS, and, subsequently, incubated for 2 h at room temperature with peroxidase-conjugated anti-human IgG (γ-chain specific) rabbit antiserum (DAKO, Copenhagen, Denmark) diluted at 1:100 with 3% milk powder in TBS. Between each treatment, the strips were rinsed for 15 min with three changes of TBS containing 0.5% Tween 20 (Sigma Chemical Co.). The reaction was visualized with 4-chlor-1-naphthol (Bio Rad Lab, Richmond, CA) in the presence of hydrogen peroxide. The total protein in the extract samples was measured by Lowry’s method [27] with bovine serum albumin (Sigma Chemical Co.) as a standard. Each sample was diluted with sample buffer to adjust the protein concentration before being applied on gel.

**RESULTS**

**Immunoblotting of Epidermal Extract (Fig 1)** On each lane of the gel, 8 μl of epidermal extract sample containing 80 μg of total protein was applied. All 28 Pw sera showed a reactivity with a single protein having an apparent molecular weight of 130 kD (lanes 1–6). In contrast, six of 23 Pf sera tested reacted with a protein of approximately 150 kD (lanes 7–10). The remaining 17 Pf sera did not react with any specific protein band (lanes 11 and 12). None of the 15 control sera showed either the 130-kD or the 150-kD protein (lane 13).

**Immunoblotting of Desmosome Preparation (Fig 2)** For this experiment, a desmosome sample (6 μg protein/lane) and epidermal extract (80 μg/lane) were run side by side in the same gel, and strips blotted with a pair of samples were stained with the same serum simultaneously. Sera from 16 Pw patients, 13 Pf patients, and five controls were tested. Eight of 13 Pf sera showed a 150-kD band in the desmosome sample (lanes 1, 3, and 5), while only three of these eight positive sera were reactive with the 150-kD band in the epidermal extract (lane 6). Five Pf sera did not show any specific band in either sample. All 16 Pw sera clearly demonstrated a 130-kD protein in the epidermal extract (lanes 8, 10, and 12), while 8 showed a slightly larger 135-kD protein band in the desmosome.
sample (lanes 7, 9, and 11). Furthermore, four Pvs sera reacted with a 150-kD band in the desmosomal sample, which had an identical mobility to the protein identified by Pf sera (lanes 7 and 9). None of the five control sera yielded the specific bands in either the epidermal extract or the desmosomal preparation.

**Immunoblotting of Cultured Squamous Carcinoma Cell Extract (Fig 3)** In this experiment, extract from cultured cells (16 μg/lane) was applied side by side with the epidermal extract (80 μg/lane) and stained simultaneously. Sera from 12 Pvs patients, nine Pf patients, and five controls were tested. The IIF test revealed a positive cell surface staining by all 12 Pvs sera. However, the KU8 cells were stained by only five of nine Pf sera.

On immunoblot analysis, all 12 Pvs sera strongly reacted with the 130-kD protein in the epidermal extract (lanes 5, 7, 9, and 11). However, in the squamous cell carcinoma cell extract, seven Pvs sera showed a clear 130-kD band (lane 6), three showed a faint band (lanes 8 and 10), and two did not react with any band (lane 12). In the squamous carcinoma cell extract, no specific protein was demonstrated by any of the nine Pf sera (lanes 2 and 4) which showed the 150-kD protein in the epidermal extract (lanes 1 and 3) or in the desmosomal sample. None of the five control sera yielded the specific bands in either sample.

**Immunoblotting of Epidermal Extracts Using Modified Extraction Procedures (Fig 4)** To determine the reason Stanley and co-workers could not detect Pvs antigen on immunoblotting [7–15], we performed two sets of experiments using different extraction procedures.

In each set, the epidermis (750 mg) separated from one foreskin by dispase was divided into three equal portions. Each portion was extracted with 1 ml buffer by different procedures described below, and the extract was diluted 1:4 with the sample buffer before the application to the gel. The final amount of the total protein loaded in each lane was about 240 μg for all extracts. In each experiment, the three different extracts were simultaneously stained with one of the four selected Pvs sera.

In the first experiment (Fig 4, top), one epidermis portion was processed by our standard method described above. All Pvs sera showed a strong reactivity with the 130-kD Pvs antigen in this sample (lanes 1, 4, 7, and 10). The second portion was extracted by the same method, but 2-ME was deleted from the extraction buffer. In this sample, all Pvs sera clearly showed the Pvs antigen (lanes 2, 5, 8, and 11). The third portion was homogenized in the same buffer as that for the standard method, but the homogenate was centrifuged at 15,000 X g for 30 min before boiling. The supernatant was then boiled and loaded on the gel. Even in this sample, all Pvs sera demonstrated the Pvs antigen at almost the same intensity as that of the standard method (lanes 3, 6, 9, and 12).

In the second set of experiments, all extraction procedures were performed in the absence of 2-ME (Fig 4, bottom). The first portion of epidermis was extracted by the standard method except for the absence of 2-ME. This was the same procedure as the second one in the first experiment. All Pvs sera again yielded the clear 130-kD band in this sample (lanes 1, 4, 7, and 10). The second portion of epidermis was extracted with both 2-ME and proteinase inhibitors deleted from the extraction buffer. Removal of these reagents resulted in considerable reduction of the reactivity, although the Pvs antigen was still seen (lanes 2, 5, 8, and 11). The third portion was homogenized in the extraction buffer containing all proteinase inhibitors but not 2-ME, and was centrifuged before boiling. The supernatant was then boiled and applied on the gel. The reactivities of all Pvs sera with the Pvs antigen in this sample were negative or extremely weak (lanes 3, 6, 9, and 12). No specific band was demonstrated even if a twofold or fourfold amount of this sample was loaded (lanes 13 and 14). All results described above are summarized and depicted in Table I.
**Table I.** The Effect of Heat Treatment, 2-ME, and Proteinase Inhibitors on the Liberation of Pv Antigen from Human Epidermis

<table>
<thead>
<tr>
<th>Heat treatment</th>
<th>Reduction with 2-ME</th>
<th>Proteinase inhibitors</th>
<th>Demonstration of Pv antigen by immunoblotting</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)*</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>(2)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(3)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(4)</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>(5)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Our standard extraction procedure.

**DISCUSSION**

Our immunoblot study revealed that all 28 Pv sera were reactive with a 130-kD protein in human epidermal extract. This protein seemed to be comparable with a 130-kD protein from mouse- and human-keratinocytes demonstrated by immunoprecipitation by Stanley and co-worker's [7–15] and with a 140-kD protein from bovine tongue desmosome demonstrated by immunoblotting by Jones et al [16].

Stanley et al were not able to demonstrate Pv antigen by immunoblot analysis of human epidermis derived from suction blister roofs. The extraction buffer they used contained neither 2-ME nor all the proteinase inhibitors used in our technique. They harvested the supernatant of the homogenate before boiling.

To clarify the reason for the inability to detect Pv antigen by their method, we first examined epidermal samples which were extracted either in the absence of 2-ME without heat treatment, and compared them to our standard technique. Every Pv serum demonstrated the Pv antigen in all three samples in almost the same intensities. The second set of experiments showed that the absence of proteinase inhibitors considerably reduced the reactivity of Pv sera with the Pv antigen. Furthermore, the 130-kD protein was virtually absent when the supernatant of the unboiled homogenate in the buffer without 2-ME was used.

These results indicate that either the heat treatment or the full reduction with 2-ME is necessary to liberate a sufficient amount of Pv antigen from epidermis. The presence of proteinase inhibitors did not seem to be essential to detect the Pv antigen, but might enhance sensitivity.

Six of 23 Pf sera showed a reactivity with a 150-kD protein in epidermal extract, a result similar to that reported by Stanley et al [7].

Because the close relationship between pemphigus antigens and desmosomes has been suggested [11–13, 16], we examined the reactivities of Pv and Pf sera with a bovine nuzzle desmosome preparation. Eight of 13 Pf sera tested showed a reactivity with a 150-kD protein, which was most likely desmoglein I. This result was similar to that of earlier studies [7,8,14]. Sera from eight of 16 Pv patients reacted with a 135-kD protein, a slightly less mobile molecule than that found in human epidermal extract. This may confirm the result reported by Jones et al [16], in which the Pv antigen showed a slightly higher molecular weight of 140 kD. However, they found this antigen only in a bovine tongue desmosome preparation but not in bovine nuzzle desmosome.

Furthermore, in the desmosome sample, four of 16 Pv sera showed an additional 150-kD band, which seemed to be identical to the Pf antigen. By means of the immunoprecipitation technique with an iodinated epidermal extract as an antigen source, Eyre and Stanley [15] also reported that 14 of 22 Pv sera precipitated Pf antigens. In the same report, they showed that both Pv and Pf antigens were precipitated as a complex of several polypeptides. Among them, 85-kD polypeptides precipitated by both Pv and Pf sera were identical, and a 130-kD polypeptide precipitated by Pv sera and a 160-kD polypeptide by Pf sera showed related biochemical characteristics. Our immunoblot analysis was able to demonstrate only 130-kD and 150-kD proteins as pemphigus antigens, which correspond to the molecules having antigenic sites for the anti-IgG autoantibodies. Nevertheless, the result that both 135-kD and 150-kD proteins of the bovine muzzel desmosome preparation were simultaneously recognized by some of Pv sera further shows a close relationship between Pv and Pf antigens. It is not known why Pv sera yielded the 150-kD band only in desmosome preparation, but not in epidermal extract.

Immunoblotting of extracts from cultured squamous carcinoma cells showed that the reactivities of Pv sera with the 130-kD protein were quite variable. Although the total protein loaded was about one-fifth of that of the epidermal extract, seven of 12 Pv sera showed a strong 130-kD band, and three sera showed a faint 130-kD band. Two Pv sera did not show protein bands in the extract of squamous carcinoma cells. No specific protein band was detected in the same sample by the nine Pf sera tested, although about half of the sera showed strong reactivity with the cell surfaces by IIF.

Several inconsistent results were obtained in the three different immunoblot systems for Pv and Pf antigens with respect to both the presence of antigen and staining patterns. These results are consistent with heterogeneities of the molecules or epitopes, with which Pv and Pf sera reacted.

Immunoblotting is generally easier and much less hazardous than the immunoprecipitation technique. From our results, especially the consistent detection of Pv antigen, it appears that immunoblot analysis should be a reliable technique for defining pemphigus antigens and could be a valuable tool for differentiation of Pv and Pf, a diagnostic distinction that sometimes is difficult.

We thank Yoshiko Fujii for her technical assistance and Sakae Hashimoto for her preparation of this manuscript. We also thank Dr. H. Tazaki, Department of Urology of Koto University, for providing us with the KU8 cells.

**REFERENCES**


